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THE NATIONAL HEART AND LUNG INSTITUTE

ANNUAL REPORT FY 1976

July 1, 1975 through June 30, 1976

NATIONAL INSTITUTES OF HEALTH
NATIONAL HEART AND LUNG INSTITUTE
Annual Report
July 1, 1975 - June 30, 1976
Office of the Director

The missions of the National Heart and Lung Institute are 1) to conduct and support research to increase fundamental and clinical knowledge about the cardiovascular, blood, and pulmonary systems; 2) to develop and evaluate improved methods of prevention, diagnosis, and treatment for diseases affecting these systems; 3) to encourage application of proven new techniques by the medical and research communities; 4) to provide support for the training of research workers, clinical scientists, clinicians, and teachers in the cardiovascular, blood, and pulmonary fields; and 5) to inform both the general public and health professionals about research and clinical advances developing out of Institute programs.

In May, 1976, the President signed legislation amending the Public Health Service Act to expand the Institute's authority and responsibilities, primarily with respect to blood-related activities within the Institute and also throughout the National Heart, Blood Vessel, Lung, and Blood Program. When implemented, it will also change the Institute's name to the National Heart, Lung, and Blood Institute.

Blood-related activities that will undergo some expansion as a result of the legislation include research on hemophilia and other hemorrhagic diseases; on clotting complications of heart and blood-vessel disorders; and on sickle cell disease, Cooley's anemia, and other diseases affecting red blood cells. Other activities are concerned with improving all phases of blood-resources management to insure adequate supplies of whole blood and blood products, eliminate waste and minimize losses, and to increase the safety and effectiveness of blood, plasma, or component therapy.

Some highlights of progress during the year in these and other Institute program areas are briefly described in the sections that follow.

Heart and Blood Vessel Diseases

A major concern of the Institute is to determine whether and to what extent premature illness or death from coronary heart disease and other consequences of arteriosclerosis can be reduced by interventions against modifiable risk factors known to increase vulnerability to these disorders. Three of the most potent and most pervasive of these risk factors in the U.S. population are elevated blood lipids, hypertension, and cigarette smoking. They are targets of several major clinical trials currently in progress.

--The Lipid Research Clinics Coronary Primary Prevention Trial involves some 4000 men who have Type II hyperlipoproteinemia (characterized by elevated blood cholesterol levels) but who have not yet developed overt coronary heart disease. The study is comparing the effectiveness of a cholesterol-lowering diet plus placebo with the same diet plus the cholesterol-lowering drug cholestyramine in reducing the threat of heart attacks or other clinical manifestations of coronary heart disease. This seven-year trial is underway at 12 Lipid Research Clinics: 11 in the U.S. and one in Canada.

--The Hypertension Detection and Follow-up Program involves 14 participating centers and approximately 11,000 patients with high blood pressure. These were randomly assigned either to the referred-care group, who are treated by their regular physicians, or the stepped-care group, treated at study clinics employing a highly systematized approach to therapy. Both groups will be followed for five years to assess the impact of adequate blood-pressure control on morbidity and mortality from cardiovascular diseases.

--The Multiple Risk Factor Intervention Trial has completed recruitment of nearly 13,000 men, aged 35-57, at heightened risk because of various combinations of elevated blood lipids, elevated blood pressure, and/or cigarette smoking. Half have been allocated to a special intervention program directed against these three risk factors, the other half referred to their usual source of medical care. The goal is to determine whether systematic modification of these three risk factors over a six-year period can reduce mortality from coronary heart disease by as much as 50 percent.

Other Institute-supported clinical trials are concerned with evaluation of techniques for secondary prevention or promising new therapeutic procedures in patients who have already been stricken by clinical manifestions of coronary heart disease. These include:

--The Aspirin-Myocardial Infarction Study, to assess the value of aspirin--an inhibitor of platelet aggregation and, we hope, of clotting complications of heart and blood-vessel diseases--in reducing the threat of recurrent heart attacks among persons who have already experienced one or more. The three-year study involves 30 participating centers and 4,200 patients.

--The Coronary Artery Surgical Trial will compare results obtained with coronary artery bypass surgery with those obtained with more conventional medical management of angina pectoris and other forms of chronic coronary heart disease. Information will be obtained both from an extensive patient registry and from a series of 600-1000 patients participating in controlled clinical trials of the two

approaches. The goal is to define the indications, advantages, limitations, and long-term benefits of a surgical procedure currently performed on some 50,000 Americans a year.

Institute supported studies indicate that the extent and severity of heart damage after an acute heart attack may be limited or reduced by the timely use of measures to protect threatened areas of heart muscle against the destructive effects of blood deprivation. Among those showing promise for this purpose have been nitroglycerin and other agents that reduce heart work (and hence its oxygen needs); glucose-potassium-insulin infusions or other measures that facilitate the entry of nutrients or correct local electrolyte disturbances in ischemic areas; and measures to reduce inflammation and breakdown of blood-deprived tissues. Proceeding concurrently with this work has been the development of more reliable, sensitive techniques for accurately defining the infarcted area and for assessing the severity of ischemic damage.

Disturbances in heart rhythm are an ever-present threat during the first few days after an acute heart attack. In some patients, this threat may persist throughout recovery and beyond, placing the patient at high risk of sudden cardiac death. Monitoring electrocardiograms, especially late in the hospital course, helps to identify these high-risk patients, and chronic administration of beta-blocking agents appears to confer some protection against potentially fatal arrhythmias.

Hypertension, though perhaps the easiest cardiovascular disease to detect and one that can readily be controlled by available therapeutic measures, nevertheless often goes undetected and either untreated or poorly controlled. To combat this problem, the Institute has participated since 1972 as coordinating agency for the National High Blood Pressure Education Program. Goals of this Program are to alert health professionals and the general public to the wide prevalence of hypertension, the insidious nature of the disease, the dangers of untreated hypertension, the availability of effective means for controlling the disease, and the health benefits of adequate therapy.

The Program carries out and sponsors a wide variety of activities in education, research, planning, and community service. That these efforts are bearing fruit is indicated by a recent upsurge in office visits for hypertension and in prescriptions written or refilled for antihypertensive drugs. Recent survey data indicate that, since the Program began operations, the number of people with unsuspected high blood pressure has been sharply reduced and the number receiving adequate treatment for their hypertension has nearly doubled.

Lung Diseases

Lung disorders falling under the purview of Institute programs afflict some 14 million Americans, are a major cause of illness and disability, cause nearly 46,000 deaths a year, and cost the economy more than $6 billion annually in lost productivity, lost wages, and medical expenses.

In seeking more effective means of dealing with these disorders, a major objective has been clarification of fundamental relationships between lung structure and lung function and delineation of changes in both that may occur with the development of disease.

Recent developments in cell-culture techniques promise to make possible detailed *in vitro* studies of some of the nearly 40 different types of cells found in the lung, including determination of their specific functions and their responses to various insults thought to operate in the development of lung diseases or to aggravate their clinical course.

Viable, homogenous preparations of endothelial cells, fibroblasts, macrophages, and type II alveolar cells are now available for such studies. They have already provided valuable clues to the source of the surfactant material that is neccessary for preventing collapse of the respiratory units, or alveoli, of the lung (the type II cells may produce it). A deficiency of this surfactant is a critical factor in respiratory distress syndrome, or hyaline membrane disease, a leading cause of death among newborn infants.

The prenatal identification of infants likely to develop respiratory distress syndrome during the neonatal period may be facilitated by two recent developments. Surfactant is present in the amniotic fluid that surrounds the fetus. Scientists have purified protein components of this material and have developed specific antibodies against them that provide a rapid reliable, and cheap means of assaying even minute amounts of the amniotic-fluid surfactant. Though not yet clinically evaluated, the techniques may prove useful for prenatal detection of surfactant deficiencies that increase susceptibility to RDS in the newborn. Forewarned, obstetricians could then arrange for the delivery of threatened infants in medical centers with facilities and staff to deal with this otherwise often-fatal disorder.

Although environmental factors, such as smoking and air pollution, are strongly implicated in the development of emphysema, some people are genetically predisposed to development of this disorder by a hereditary deficiency of alpha-1-antitrypsin. This enzyme combats protein-digesting substances, collectively called proteases, released in the lung by macrophages in the course of their battles with bacteria and other invaders. Unless neutralized, these byproducts of the lung's defenses can themselves damage lung tissues.

The results of recent research on alpha-1-antitrypsin and its variants promises to improve the reliability and sensitivity of tests to detect hereditary deficiencies, perhaps enabling those affected to escape

potential consequences of their heightened vulnerability. Such research may also lead, in time, to direct means of combatting such deficiencies or lung damage resulting from them.

So insidious is the development of emphysema and certain other chronic lung disorders that, by the time the victim becomes aware of the symptoms, much irreparable lung damage may already have occurred. Hence the emphasis of Institute programs on development and evaluation of methods for diagnosis of these diseases in their earliest, often asymptomatic, stages.

An x-ray technique called microfocal spot magnification, though not yet tested clinically, appears to have high potential for use in early diagnosis. It permits visualization of the smallest airways and other tiny lung structures without any significant increase in radiation exposure. This technique may soon enable physicians to study in some detail the behavior of the smallest airways during respiratory function tests. These structures appear to be affected earliest by chronic obstructive lung diseases, and at this early stage the disease process may well be reversible.

Blood Diseases and Blood Resources

Institute-supported research on hemorrhagic diseases continues its emphasis on hemophilia, a disorder that imposes a drain on the national blood resource out of all proportion to the number of people afflicted (the equivalent of some 3 million units of whole blood each year for the episodic care of some 20,000 people with severe hemophilia).

Recent research has shown that hemophiliacs do not have an absolute deficiency of antihemophilic factor (AHF), but rather produce an abnormal variant of this essential clotting factor that participates poorly or not at all in the coagulation of blood. At present, the only means of correcting (temporarily) the coagulation defect in hemophiliacs is by providing normal AHF extracted from fresh whole blood or plasma. Both starter substances are expensive; and the AHF concentrates are even more expensive, since the costs of processing and presently unavoidable AHF losses incurred in preparing the concentrates are both determinants of the price per unit.

The AHF molecule is so large and complex that laboratory synthesis appears unlikely. But the procoagulant activity of AHF appears to reside in a smaller subunit or fragment of the molecule, so that the eventual production of a synthetic substitute that performs the same function as the normal clotting factor is a possibility that continues to be actively investigated. Meanwhile, the Institute also supports research concerned with improving the yield of currently available AHF extraction procedures and with developing better ones that may make AHF concentrates more widely available at lower cost.

And what has already been learned about normal and abnormal AHF has already provided means that, according to the investigators, can identify female carriers of hemophilia with a high degree of accuracy before they give proof

of their carrier status by bearing hemophilic sons. Their method takes advantage of the fact that carriers produce both normal AHF and abnormal variants, the first measurable by regular coagulation tests, the second by recently developed immunochemical techniques. They claim that a comparison of both tests has given 90 percent or better accuracy in detecting hemophilia carriers.

Institute-supported research on sickle cell anemia has included evaluation of anti-sickling agents that might prevent or shorten painful, disabling sickle-cell crises that occur in unpredictable fashion in victims of the disease. A number of antisickling agents have been studied, but several of the more effective ones have had toxic side effects that severely restricted their clinical use. The quest continues for safe, effective agents suitable for oral use or intravenous infusion. Other possibilities under investigation include use of extracorporeal circulation techniques to maximize the antisickling action of effective, but toxic agents while minimizing their adverse side effects.

Other research is concerned with prenatal detection of sickle cell anemia and related disorders by studying red blood cells obtained from the fetus or amniotic fluid during pregnancy. The tricky part is obtaining the red cells without hazard to mother or fetus, though recent developments in fetoscope design promise solutions to this problem.

Victims of Cooley's anemia (Thalassemia) have a genetic flaw in hemoglobin production that results in premature destruction of their own red blood cells. Repeated transfusions are needed to sustain normal RBC levels, and these cells too have a reduced life expectancy. The accelerated RBC destruction in patients with Cooley's anemia leads, in time, to the accumulation in various organs and tissues of excessive iron compounds which the body can neither tolerate well nor readily excrete. It is this iron overload that is usually responsible for disabling or lethal consequences of the disease.

The search has continued for safe, effective iron-chelating agents that might relieve this iron overload by converting iron compounds into forms more readily excreted by the kidneys. The best agent evaluated thus far has been Desferrioxamine, which appears to be a safe and potent drug for promoting the excretion of excess iron accumulating in the liver.

One of the most worrisome problems of therapy with blood products is the risk of transfusing hepatitis virus along with the needed blood or blood component. Present techniques for identifying hepatitis in blood products are effective less than half the time, nor can blood products so identified be reclaimed for clinical use by eliminating the offending virus.

Recent clinical results indicate that temporary protection against hepatitis B may be conferred on high-risk subjects by injections of hepatitis B immune globulin (HBIG). This helps, though hepatitis B is only one facet of the overall problem of transfusion hepatitis; others are hepatitis A and and non-A non-B hepatitis, possibly more common than the other two. Current research continues to seek solutions to all of these problems.

Intramural Research

The Institute's Intramural Research Program, carried out in federal laboratories at Bethesda, represents in microcosm the varied research and clinical interests of the research support programs. Among the results reported by intramural scientists during the past year were the following:

--Echocardiography is a safe, painless, non-invasive technique employing reflected ultrasound to visualize heart structure or to evaluate heart function. An extremely versatile diagnostic technique, it had previously demonstrated its value in detecting and evaluating various cardiomyopathies and congenital heart defects.

Now Institute scientists report that echocardiography can detect early, often asymptomatic alterations in heart structure and function that may progress to congestive heart failure in chronic alcoholism. It is effective in detecting and monitoring cardiac deterioration that may occur with the gradual deposition of excess iron in the heart tissues of patients with Cooley's anemia. And it can be used to detect and evaluate enlargement of the left atrium, which may lead to the development of atrial fibrillation, a common and important disorder of heart rhythm, with its attendant danger of thromboembolic complications. All three applications have potentially important implications in prevention and therapy.

--Animal studies by Institute scientists showed that nitroglycerin or nitroglycerin plus methoxamine clearly outperformed several other drug regimens that have been proposed for limiting or reducing heart-muscle damage after acute heart attacks. Other regimens tested included phentolamine or nitroprusside, both powerful blood-vessel dilators thought to improve collateral bloodflow to heart muscle and to reduce the cardiac workload, and mannitol, an osmotic diuretic thought to curb swelling or disruption of blood-deprived heart tissues and to facilitate the penetration of nutrients into ischemic areas.

Only nitroglycerin or nitroglycerin plus methoxamine consistently increased collateral bloodflow to the heart and also reduced heart-muscle damage, confirming the results of earlier animal studies and also the promising early results of ongoing clinical studies in heart-attack patients.

--Some scientists elsewhere have hypothesized that stress may be an important component in hypertension and have reported that patients can be trained to bring their elevated blood pressure down by training in relaxation techniques, yoga, or related so-called "bio-feedback" techniques. Institute scientists evalulated these techniques in 22 patients with borderline to mild hypertension, but found that they had no significant effects on blood pressure.

--Alpha-methyldopa is a drug widely used in the treatment of hypertension. In most patients it is safe and effective. But about one-third of those taking the drug give evidence of mild liver dysfunction or damage and, in a much smaller number, there is evidence that the drug may initiate a chronic, active hepatitis that is occasionally fatal.

Studies by Institute scientists suggest that the liver damage may stem from a chemically active drug metabolite generated by the interaction of alpha-methyldopa with a product of an electron transfer particle, called cytochrome P-450, found in liver microsomes. These microsomes are tiny organelles that play a major role in the inactivation of drugs and other "foreign" compounds and also in their excretion. (In general, they convert these compounds into more polar substances that are more easily handled by the kidneys.) But occasionally these microsomes generate drug metabolites that are more active or more toxic than the parent drug.

In the case of alpha-methyldopa, the Institute scientists' scenario goes like this: In the presence of oxygen, cytochrome P-450 forms a complex that subsequently dissociates into oxidized cytochrome P-450 plus a highly reactive superoxide radical. The radical then reacts with alpha-methyldopa, converting the drug into a chemically active metabolite that binds firmly to various protein, enzyme, or other components of liver cells, interfering with their functions and sometimes causing the death of affected cells. A similar mechanism has been postulated for the liver toxicity occasionally observed with other drugs, including the aspirin substitute acetaminophen and the diuretic agent furosemide.

Other Institute Activities

The Institute continues to cooperate with Soviet scientists as part of the US-USSR Health Exchange Program initiated in 1972. Major areas of collaborative research include: 1) pathogenesis of arteriosclerosis; 2) management of ischemic heart disease: 3) myocardial metabolism; 4) congenital heart disease; 5) sudden cardiac death; 6) blood transfusion and hepatitis, particularly with respect to cardiovascular surgery; and 7) artificial heart research and development.

Under this program, there has been continued exchange of American and Soviet health researchers as well as productive planning sessions held here or in Russia. In a number of areas, the program has moved from the planning phases into ongoing basic or clinical studies, although there is little to report from these as yet.

New Institute staff appointments during the year included the following:

--Robert I. Levy, M.D., as Institute Director, replacing Dr. Theodore Cooper, M.D., who left NHLI to assume the post of Assistant Secretary

for Health, DHEW. Prior to his appointment as Institute Director, Dr. Levy had served as Director of the Division of Heart and Vascular Diseases.

--Sidney Blumenthal, M.D., as Director of the Division of Heart and Vascular Diseases, replacing Dr. Levy. Before coming to the Institute, Dr. Blumenthal was Professor of Pediatric Cardiology at the University of Miami School of Medicine.

--Wolf W. Zuelzer, M.D., as Director of the Division of Blood Diseases and Resources. He was formerly President and Co-Director of the Michigan Blood Center, as well as Professor of Pediatric Research at Wayne State University and Director of the Child Research Center of Michigan.

--Clarice D. Reid, M.D., as Chief of the Sickle Cell Disease Branch. She replaces Rudolph E. Jackson, M.D., who continues to serve the Institute as a special consultant to its Molecular Hematology Branch. Before coming to NHLI Dr. Reid was a medical officer with the DHEW Bureau of Community Health Services and Deputy Director of its SCD Program.

--Ronald G. Crystal, M.D., as Chief of the Pulmonary Branch in the Institute's Division of Intramural Research. He had formerly headed that Branch's Section on Pulmonary Biochemistry.

--Ruth Hegyeli, M.D., as Assistant to the Director, NHLI, for International Programs. She had formerly served as Chief of the Program Development and Evaluation Branch in the Institute's Office of Program Planning and Evaluation, and also as Acting Director of that office.

--Jay Moskowitz, Ph.D., as Director of the Office of Program Planning and Evaluation, replacing Dr. Hegyeli. Dr. Moskowitz formerly held a similar post in the Division of Lung Diseases.

--Ms. Evelyn Attix, as Director of the Institute's Office of Administrative Management. She had formerly served as Administrative Officer for the Division of Intramural Research.

--Mr. James M. Pike, as Chief of the Grants Operation Branch in the Institute's Division of Extramural Affairs.

Annual Report
Office of Prevention, Control and Education
National Heart and Lung Institute
July 1, 1975 through June 30, 1976

OPCE

The Office of Prevention, Control and Education, a staff office within the Office of the Director headed by the Assistant Director for Health Information, was established in response to a Congressional mandate directing the NHLI to become involved in the translation of biomedical research into health practices for the prevention and control of heart, lung and blood disorders. Given limited funds, the Institute decided to focus on prevention and control through education and educational research. OPCE was given the authority and responsibility for support and coordination of PCE programs throughout the Institute. OPCE reviews all NHLI/PCE programs prior to implementation, renders technical and program assistance to the Divisions, identifies areas of potential cooperation among Divisions as well as future educational research areas within current basic and clinical research program segments, and develops cooperative relationships with external agencies and organizations as are beneficial to the development of NHLI/PCE programs.

During the past year, the OPCE mission has been executed through a number of activities undertaken by OPCE itself and its two branches, the Health Education and Behavioral Research Branch and the Public Inquiries and Reports Branch. OPCE has, for example, initiated or conducted projects related to continuing medical education and the development of a conceptual model of normative work flow of the Institute to be used as a program planning tool.

As part of OPCE, the Health Education and Behavioral Research Branch is responsible for bringing behavioral/social science research capabilities to bear on relevant program areas of the NHLI and its Divisions. In addition, it is responsible for acquainting Institute staff with the capabilities of behavioral and social sciences in resolving health behavior problems, and for stimulating research relative to NHLI within the behavioral/social science communities.

During the past year, a number of activities were initiated or continued by this Branch. Among other things, these activities were related to implementation of a grants tracking system to monitor behavioral studies sponsored by the Institute, development of "state-of-science" information resource in such areas as non-pharmacologic approaches to the treatment of hypertension, expansion of a behavioral/ educational consultant roster, continuation of the Visiting Scholar Program, and cooperation with other Institutes and other organizational entities outside NIH sharing educational and behavioral research concerns for prevention and control of heart, lung and blood diseases.

Responsible for fulfilling the traditional role of an information office, the Public Inquiries and Reports Branch serves as focal point for

the dissemination of information and education materials to health professionals, scientists and the general public through established and innovative marketing techniques. The Branch annually responds to over 60,000 inquiries and distributes in excess of 1.3 million publications reporting on scientific workshops, symposia, task force findings and published research.

This fiscal year, the Branch, in cooperation with the Institute's scientific staff, completed a needs assessment of clinical trials to establish an orderly flow of information resulting from the investigations; continued a public affairs trends analysis, tabulating data on the mechanisms of dissemination and resulting impact; strengthened relations with private and voluntary associations and institutions through participation in workshops and conferences; established and maintained a vigorous training program for employees in information skills, creating the Institute's first Retraining Position.

The Branch also began cooperative efforts with other components of NIH, evaluating the impact of information/education materials on mass media and identifying "gatekeepers" controlling access to the media, alerting them to the significance of cardiovascular, pulmonary and blood research. Communications skills were applied to recruitment phases of several clinical trials, ultimately enabling the Institute to evaluate progress through effective recruitment.

ANNUAL REPORT OF THE DIVISION OF HEART AND VASCULAR DISEASES, NHLI

JULY 1, 1975 - JUNE 30, 1976

1. General Mission of the Division of Heart and Vascular Diseases

The Division of Heart and Vascular Diseases (DHVD) is responsible for planning, directing and conducting the National Heart and Lung Institute's research grant, contract and training programs in heart and vascular diseases. These programs encompass basic research; targeted research; clinical trials; and education, demonstration and control activities. The Division maintains surveillance over developments in its program areas and assesses the national need for research in the causes, prevention, diagnosis and treatment in these disease areas. The scope of the Division's mission is indicated by the ten research areas which have been identified in the Institute's National Plan and Program: Arteriosclerosis, Hypertension, Cerebrovascular Disease, Coronary Heart Disease, Peripheral Vascular Diseases, Arrhythmias, Heart Failure and Shock, Congenital and Rheumatic Heart Diseases, Cardiomyopathies and Infections of the Heart, and Circulatory Assistance.

There has been in recent years, a slight decrease in the death rate in coronary heart disease, but cardiovascular disease continues to be the number one killer in the United States. A major focus of the Division is on arteriosclerosis and hypertension which are underlying factors in over 1,000,000 deaths in this country every year.

In studying the ten research areas of the National Plan the Division uses a broad based approach, employing all available funding and administrative mechanisms to take action to support (1) new basic research, which continues to be the Division's highest priority; (2) clinical evaluation of promising research findings and concepts through clinical trials; and (3) education, demonstration and control programs such as the National High Blood Pressure Education Program, which translate research concepts to practical patient care.

2. Progress Toward Meeting Objectives of the Division of Heart and Vascular Diseases

A major objective of the Division is to increase the knowledge and understanding of the basic mechanisms and processes underlying and leading to the cardiovascular diseases under study. Progress in these basic research areas is illustrated by a number of examples (selected from a much larger number) of research findings summarized in the following section, Highlights of Accomplishments.

Activities in the Division's targeted areas are proceeding according to schedule. The following summary of the status of the Division's major Clinical Trials and other activities provides indication of progress toward meeting a number of the designated objectives of the Division.

The Aspirin-Myocardial Infarction Study: This new study of the efficacy of aspirin in reducing mortality and recurrent myocardial infarctions in men and women with coronary heart disease has finished recruiting 4200 patients. The recruitment of patients into this study was accomplished within the one year planned for enrollment. This achievement, unusual in collaborative clinical trials, was due to the experience of many of the investigators and to the simultaneous use of several recruitment techniques. Follow-up of the patients is scheduled to continue until summer of 1979.

Multiple Risk Factor Intervention Trial: The primary objective of the MRFIT is to determine whether, for a group of men at above average risk of death from coronary heart disease, a special intervention program for six years will result in a 50% reduction in mortality from coronary heart disease. To achieve this objective, over 360,000 men were screened to identify 12,866 men age 35-57 years who are in the upper 10 percentile at elevated risk for death from coronary heart disease based on risk factors of elevated serum cholesterol, elevated diastolic blood pressure and cigarette smoking, and whose risk factors are such as to permit potential major reduction in risk by altering risk factors. Of the men selected, half were randomly allocated to a Special Intervention program and half were referred back to their physicians or their usual source of medical care. The intervention program is designed to achieve and maintain a lowering of serum cholesterol, a reduction in blood pressure and cessation of cigarette smoking.

The results in cigarette smoking intervention appear to exceed the experience of previous smoking cessation programs. However, relapse is a substantial problem and MRFIT's successes will need reinforcement to maintain these results. Hypertension intervention is progressing adequately; although the reductions assumed by the study design have not been reached, it is anticipated that they will be. The most serious intervention lag relates to nutrition; the reductions here have fallen short of those projected by the study design. Instead of an estimated 10% difference between Special Intervention and Usual Care, the difference is about 3% at the first annual exam. The investigators are well aware of the problem and are taking steps to improve the situation. Additional staffing for nutrition intervention and additional training for nutritionists to help improve their facilitating role is being sought.

Hypertension Detection and Follow-Up Program (HDFP): The HDFP is designed to determine the effectiveness of a systematic approach of antihypertensive therapy in reducing morbidity and mortality in men and women with elevated blood pressure in community-based populations. In February 1973, the HDFP initiated screening and follow-up for high blood pressure in 13 geographically defined communities and in one selected industrial community. By June 1974, approximately 160,000 men and women age 30-69 were screened for elevated blood pressure. Approximately 11,000 hypertensive participants were randomized to either Stepped Care or Referred Care. Stepped Care is a series of carefully controlled drug regimens for participants who are assigned to HDFP clinics for their hypertensive therapy. Persons not selected for Stepped Care are referred to their own physician for treatment and are the Referred Care Group. During the past year, there have been nearly 35,000 follow-up clinic visits on Stepped Care participants, 9860 second year home visits and 8872 second year clinic examinations on both Stepped Care and Referred Care participants. At the first year home visits, the mean diastolic blood pressure dropped 13.8 mm Hg from baseline for the Stepped Care participants and 7.2 mm Hg baseline for the Preferred Care participants. The mean systolic blood pressure dropped 26.6 mm Hg for the Stepped Care participants and 14.1 mm Hg for the Referred Care participants during this same time period. 81% of Stepped Care participants reported they were taking their medicine as compared to only 52% of Referred Care participants.

Coronary Artery Surgery Trial (CAST). This study seeks to assess the effects of coronary artery bypass graft surgery on morbidity and mortality in patients with chronic coronary heart disease through the establishment and analysis of a patient registry with approximately 20,000 patients and through randomized studies of 600 to 1000 patients randomized into surgical and medical therapy groups. This Trial has gone into its definitive stage. Approximately 8,000 patients have entered into the registry, and approximately 100 into the much more limited randomized series to which recruitment was begun much later. The number of participating institutions in the Trial has been increased from the original 11 clinical centers to 16, with the addition of 5 new ones in June, 1976. Recruitment of patients will continue through June 1978 with an estimated 600 to 1000 patients in the randomized series to be followed for five years.

Lipid Research Clinics Coronary Primary Prevention Trial (LRCCPPT)

This trial, commenced in 1973 is a seven-year study to test whether lowering cholesterol in hypercholesterolemic otherwise healthy subjects will reduce or slow the development of premature coronary

heart disease. One of the most common of the blood lipid disorders, Type II hyperlipoproteinemia, is being studied in this trial. It is one of the most dangerous of the lipid disorders because of the associated high risk of premature atherosclerosis. Patients with this disorder are at high risk of death from coronary heart disease at a rate 2.5 to more than 10 times that of normals. Heart attacks in men under 50 with the Type II disorder are common. Twelve lipid clinics, eleven in the United States and one in Canada, are cooperating in this seven-year trial. About 450,000 age-eligible men have been screened to identify the required 4000 men who have an elevated blood cholesterol in the absence of severe hypertension, diabetes, endocrine disease, or other life-threatening disorders. They must be free of overt coronary heart disease or a history of heart ailments. The experimental group receives a cholesterol-lowering diet plus the cholesterol-lowering drug, cholestyramine. The control group receives the diet together with a placebo (inactive pill). Randomization will be completed in the summer of 1976.

Essential to the accomplishment of the Division's objectives is the availability of an adequate number of well trained and qualified investigators in a wide range of disciplines, from basic biologists to epidemiologists to behavioralists. In a number of important areas there are significant shortages, both in the present numbers and in the training "pipeline" which supplies the investigators needed in the near future, both for replacement and for concentration in new and expanding areas of research related to the mission of the Division. Within the policy and budgetary limits existing at present the Division has provided support for some training programs and for some individual fellowships and traineeships. The high incidence of hypertension in the black urban population poses a specific problem in the area of manpower training. The scarcity of black investigators and instructors impairs communication with the population at risk and is so severe that conventional training recruitment programs are ineffective. Individuals are needed to conduct basic and clinical research and to collaborate with primary care physicians and with community units in the Division's hypertension prevention, control and education program. In response to this identified need the Division is initiating a program to provide summer research opportunities for minority school faculty and graduate students.

The Division continues to make progress in its prevention, control and education program. The Division's main thrust in this area is the National High Blood Pressure Education Program, launched in 1972. There are a number of indications that changes have taken place in public awareness and professional behavior relevant to hypertension, in part attributable to the activities and efforts of the many private and local government agencies cooperating in this national program, coordinated by the Division. The National Health Examination Survey of 1960-62 and the National Health and Nutrition

Examination Survey of 1971 both indicated that nearly half of 23 million Americans estimated to have high blood pressure were unaware that they had it. More recent data from the Division's Hypertension Detection and Follow-Up Study (which may not be entirely applicable to the whole population) indicate that that number has decreased to 29%. In the earlier surveys it was found that about four million of the known hypertensives had their disease under adequate control. The HDFP Study, if applied to the whole nation, indicates that the number may have nearly doubled. For the same period, data from the National Disease and Therapeutic Index show a steep increase in the number of total patient visits to physicians for hypertension and hypertensive heart disease (up 50% over 1971) while total physician visits for all causes increased only 14%.

Nutritionists in DHVD continue to play an important role in the conduct of two of the Division's Clinical Trials, MRFIT and the Type II Coronary Primary Prevention Trial. Working with the nutritionists at the clinical centers of these trials, they have developed a variety of tools and techniques for use in evaluating food intake and/or adherence to diet. Division nutritionists assisted in the revision of the American Diabetes Association and the American Dietitic Association's publication "Meal PLanning with Exchange Lists" for use in nutrition counselling; the purpose of the revision is to update the information concerning food composition and to provide an improved aid for patient education. They have assisted the American Heart Association's Committee on Atherosclerosis and Hypertension in Childhood in developing educational material for teenagers on lipid lowering diets.

3. Highlights of Accomplishment

As indicated in the previous Section, progress is being made in the quest for better and more comprehensive understanding of the basic mechanisms and processes that underlie and lead to cardiovascular disease; such understanding is a basic and high priority objective of the Division. This section summarizes a small number of examples of findings that have been made in the Division's programs in laboratory, animal, clinical and epidemiological research.

An unusual renin-like enzyme having a higher molecular weight than normal plasma renin was reported last year. This new kidney-based substance, called big renin, has now been extracted and purified. Current theories about the role of renin in blood pressure regulation are based on the belief that renin exists only in active form. The recent discovery of big renin (MW 60,000 compared to normal renin MW 40,000) from hog kidney extracts indicates that the enzyme exists almost entirely as a larger molecule which is a less active or "latent" form of renin. This observation raises questions about how "big renin" is converted into the smaller, active form and what role this transformation may have in the blood pressure regulating process. Any one

of several aspects of the conversion of the inactive renin to the active renin--such as the speed and site of the conversion as well as the biochemical mechanism of change--may have some role in the development of high blood pressure. In addition, an even larger molecule of renin (MW 140,000) called "big, big renin," has been isolated. The introduction of these new elements into the already complex renin-angiotensin-aldosterone system will likely open new approaches to the study of renin and blood pressure control.

Pediatricians and biostatisticians in cooperation with the National Center for Health Statistics, using normal percentiles have developed pediatric blood pressure "grids." They are also preparing a monograph, which will be a resource of prevalence data and selected correlates of blood pressure in children.

NHLI-supported investigators have isolated and purified two post-heparin lipolytic enzymes which are important to the metabolism and transfer of lipids in tissues and the blood (post-heparin lipoprotein lipase and hepatic triglyceride lipase). They have made the important observation that the two enzymes, although identical in protein structure and amino-acid composition and residues, have different enyzmatic properties and are also immunologically distinct. Early evidence suggest that the differences are explained by the carbohydrate components of the enzymes.

The protective role of high density lipoprotein is attracting increasing attention. Recent cross-sectional and prospective studies indicate that high density lipoprotein levels are inversely, strongly and independently correlated with the risk of developing coronary heart disease. Biochemical investigations suggest that high density lipoprotein is involved in removing cholesterol from the arterial wall. Preliminary observation suggests that populations differ in their high density lipoprotein levels.

In the area of sudden cardiac death, studies have suggested that the chronic administration of beta-blocking agents may well be useful in reducing the the late mortality following heart attack, presumably by preventing ventricular fibrillation and reducing sudden cardiac death. Patients at high risk for sudden death following myocardial infarction are being identified with increasing accuracy; the monitoring of electrocardiograms especially late in the hospital course has been useful by means of identification of patients with serious ventricular arrhythmias. Improved emergency medical services in the community continue to be life-saving by providing techniques for resuscitation from cardiovascular collapse and ventricular fibrillation. In one community of a half million, there are now over a hundred lives saved annually by such techniques. Thirty percent of those who are found in ventricular fibrillation upon arrival of the emergency rescue squad are completely resuscitated and leave the hospital alive.

Diagnosis and assessment of ischemic heart disease by exercise testing has undergone important evolution. There is an increased realization of the limitations as well as of the value of the standard exercise tests. Other techniques of importance are coming into greater use, and their strengths and limitations are being better understood. Echocardiography is one such technique, with broad applicability, for the detection and quantification of a variety of abnormalities, ranging from asymmetrical hypertrophy of the myocardium to ballooning of the mitral valve, to disorders of ventricular movement, to estimates of the ventricular ejection fraction, and to the detection of congenital heart disease in the neonate. More satisfactory and precise techniques for measuring the amount of heart muscle damage in heart attacks include the measurement of creatine phosphokinase and its myocardium specific isoenzyme. The newly developed technique for the detection of myoglobin by radioimmunoassay permits a very highly sensitive though not specific technique for the very early detection of injured myocardium. A sensitive radioimmunoassay for fibrinopeptide A has been developed which is a far more specific and sensitive indicator of clotting than other available methods.

Advances have been noted in the clinical and basic research understanding of the prevention, assessment, and management of heart disease, and in development and evaluation of devices and technology for the furtherance of these goals. There has been a general decline in mortality from heart disease, and specifically for ischemic heart disease, over the past decade. The duration of hospitalization for acute myocardial infarction continues to diminish.

Coronary bypass surgery remains a major element in the management of ischemic heart disease. Recent studies have shown that for one specific anatomical lesion - severe narrowing of the left main coronary artery - prognosis is improved following the surgical procedure. On the other hand, preliminary data from the Unstable Angina Pectoris Trial show that the mortality for operative vs medical management is equal - and low. The data are now under continued observation, and definitive results warranting cessation of the trial have not been approached. This underscores the importance of the statement that coronary artery surgery cannot be considered as simply good or bad - its specific indications and long-term effects must be sharply defined. Studies to get answers to these questions are underway.

Protection of ischemic myocardium has been the focus of major research. Several promising agents, ranging from propranalol to nitrogycerin, steroids, hyaluronidase and glucose-potassium-insulin, are being studied in the clinical setting. Methods for mapping the extent of infarction which are important for doing clinical studies on a reasonable scale are being developed; a new technique in which the evolution or failure of evolution of pathological Q waves in areas over the precordium where ischemic changes in the electrocardiogram had initially evolved has been developed and is undergoing validation. Radioisotopic methods for quantifying the extent of ischemic or infarcted myocardium are being further developed. A major new technique which is suitable in chronic ischemic heart disease in contrast to acute infarction is that of noting the radioisotopically ischemic areas which develop in the course of graded exercise stress upon the heart.

4. Significant Workshops, Task Forces and Meetings

A meeting of program participants in Ischemic Heart Disease, primarily of the Ischemic Heart Disease Specialized Centers of Research (SCOR) as well as participants in the Protecting Ischemic Myocardium and Quantifying Infarct Size programs, was held February 2-3, 1976. A bound collation of the annual reports from the SCOR's is available.

A workshop to assess the possible role of chronic antiarrhythmic therapy in the reduction of sudden cardiac death was held May 6-8, 1976. The summary of its report will be available in June, 1976.

The report on the "Automated Blood Pressure Measuring Devices for Mass Screening" was published and is available as DHEW Publication No. (NIH) 76-929.

The Report of the NHLI Task Force on Genetic Factors in Atherosclerotic Diseases was issued in March, 1976. It contains recommendations of the Task Force and the written presentation made at two meetings held in December, 1974 and March, 1975. (DHEW Publication No. (NIH) 76-922).

A multi-disciplinary group of consultants met on January 29 and 30, 1976 to assist DHVD staff in assessing the needs and opportunities in relation to prevention, control and education activities in the cardiovascular area. Findings and recommendations of the group are being considered by DHVD in making plans in the area of prevention, control and education related to the Division's mission.

A meeting of consultants to discuss manpower needs in epidemiology was held May 28, 1976. Its findings and recommendations will serve as a basis for the Division's plans in this area.

The Division's Task Force on Hypertension, established to survey the entire field of hypertension research and to identify areas needing intensified or lessened emphasis, held several meetings this year. Its report is due next summer.

5. Major Problem Areas

Some aspects of the Freedom of Information and Privacy Acts represent possible problems in the Division's Clinical Trials, where premature release of data collected in the course of an ongoing study can destroy the integrity and functioning of the study.

Another problem affecting some of the Division's Clinical Trials is that of maintaining the required long-term interest of principal investigators and staff at the participating clinical centers. These studies are of long duration and their nature at times presents problems of professional and academic recognition to the participating staffs.

Definition of the role and approach the Division should take in planning and implementing its program in cardiovascular prevention, control and education has yet to be firmly established. The Division's effective program in high blood pressure education provides a partial model for application to other cardiovascular areas, but it cannot provide a complete template for other areas. Sharper focussing on the problems and opportunities and the assignment of more resources are being considered in order to establish and conduct an effective program in this Congressionally mandated and important area.

The major problem in program administration is that of recruiting appropriately qualified professional staff, because of limitations in salaries, and inadequate space.

ANNUAL REPORT

DIVISION OF BLOOD DISEASES AND RESOURCES

NATIONAL HEART AND LUNG INSTITUTE

July 1, 1975 Through June 30, 1976

The programs of the Division of Blood Diseases and Resources seek to improve the diagnosis, prevention, and treatment of blood diseases and related disorders; to assure the efficient and safe use of an adequate supply of high quality blood and blood products; and to promote the application of tissue and organ transplantation through a better understanding of transplantation biology. The programs include fundamental and clinical research, professional development and training to fill projected needs, and education, prevention and control activities to assure orderly application of the knowledge gained through research. In developing and supporting its programs the Division of Blood Diseases and Resources seeks to use all available support mechanism as appropriate. These include investigator-initiated research projects, program project grants, goal-oriented centers such as Specialized Centers of Research (SCORs), a National Research and Demonstration Center, and targeted research and development contracts. The areas covered in this report are --

1. Thrombosis and Hemorrhagic Diseases
2. Red Blood Cell Disorders and Erythropoiesis
3. Sickle Cell Disease
4. Blood Resources and Transplantation
5. Professional Development and Training

Some highlights of accomplishments of the past year are briefly mentioned below.

1. Thrombosis and Hemorrhagic Diseases

Areas of interest currently include studies on thromboembolic disorders, such as pulmonary embolism, thrombophlebitis and disseminated intravascular coagulation. The role of the hemostatic process in atherosclerosis is a topic of expanding interest. Major efforts are also being made to encourage research into bleeding disorders, with marked emphasis on the hereditary hemophilias. Disorders of platelet physiology and function are being actively pursued both from the biochemical and clinical view.

A major problem in the care of Factor VIII deficient hemophiliacs has been the occurrence of inhibitors to Factor VIII. A study of the incidence and clinical importance of these inhibitors has been implemented. It involves ten clinics with 1,200 hemophilia patients and is designed to obtain data on any important complication of hemophilia. A workshop was held under joint sponsorship of BOB/FDA, DBDR/NHLI and the National Hemophilia Foundation entitled "Unsolved Therapeutic Problems in

Hemophilia." Presentations and discussions covered such problems as hepatitis risk, cause of death, immunological problems, increased incidence of hypertension and renal disease. Publication of the proceedings is in preparation. A Hemophilia Therapy Task Force was assembled, drawing on experts from all areas concerned with management of hemophilia to discuss the optimum mode of treatment for this disease. A document suggesting possible guidelines for establishment of comprehensive hemophilia care centers was forwarded to the Health Services Administration to assist that agency in implementing a congressional mandate in this area.

Patients undergoing orthopedic surgery are particularly susceptible to postoperative thromboembolic complications. These patients have shown the least promising results with anticoagulant and antiplatelet agents. The Division is supporting four clinical trials utilizing such agents alone and in combination to clarify the status of this problem. The proceedings of a conference sponsored jointly in the DBDR/NHLI and the Council on Thrombosis of the American Heart Association, entitled "Prophylactic Therapy of Deep Vein Thrombosis and Pulmonary Embolism" was recently published [DHEW Publication No. (NIH) 76-866]. A cooperative study on Atherosclerosis and Platelet Composition, Hemostatic Function and Physiology, has been initiated with the Laboratory of Experimental Atherosclerosis, Intramural Division, NHLI. The Division supports a lipid analysis laboratory to determine the effect of diet on platelets from a unique animal model of atherosclerosis which involves extensive thrombosis. This project represents an exciting opportunity to achieve a dramatic breakthrough in our understanding of the relationship of thrombosis, platelets and atherosclerosis. A workshop was held on the "Molecular Basis of Heparin Action." Publication of the proceedings is in preparation. As a result of this workshop, a meeting of experts was assembled to discuss the preparation of a standard heparin reference, for laboratory, but not clinical use. An RFP is being prepared as a result of this meeting.

A number of major problem areas have been identified. These include, relative to hemophilia: (a) Factor VIII Biochemistry and Physiology; (b) Genetics of Factor VIII and Hemophilias; (c) Differentiation of Hemophilia and von Willebrand's Disease; Home Care vs. Center Care in Treatment of Hemophilia. Relative to Thrombosis are: (a) the role of platelets in the relationship of thrombosis to atherosclerosis; (b) the detection and treatment of high risk patients for deep vein thrombosis and pulmonary embolism.

2. Red Blood Cell Disorders and Erythropoiesis

The general mission of the program is to support fundamental and clinical research in red blood cell production, maturation and function in the normal and diseased states. Areas of interest include: (a) Cooley's Anemia (thalassemia) and iron overload; (2) erythropoiesis and stem cell kinetics; (3) hemoglobin synthesis and metallo-complexes; (4) red cell membrane structure and function; (5) cellular enzyme disorders; (6) red cell and blood rheology; and (7) oxygen transport.

The Division continues to authorize the distribution of purified urinary erythropoietin (Ep) to the scientific community worldwide. Within the past year, approximately 70,000 units have been shipped to 51 investigators, many of whom would not have been able to continue their research without this assistance. Increasing research demands, which include further purification and characterization of Ep requires an expanded, reliable domestic source of urinary Ep. An RFP entitled "Source of Human Erythropoietin" has been issued. It is anticipated that in early FY '77 a sufficient source of Ep, to meet the needs of the scientific community, will be available. The problem of iron overload, which develops as a result of chronic transfusion of thalassemia patients has necessitated a search for iron chelating agents. A panel of experts was convened to develop a position paper on the current status of a promising iron chelating agent, Desferrioxamine (Desferal^{R}). The conclusion was that Desferrioxamine is safe as well as potent and specific in removing hepatic iron from patients with excessive iron overload. The information was made available to the Food and Drug Administration to facilitate relabeling Desferrioxamine for use in chronic iron overload. The results are also being prepared for publication. Their recommendations included that further studies be undertaken to define the optimal strategy of its use, the optimal dose and route of administration, and exploration of better methods to provide sustained high dosage of the drug for prolonged periods. An RFA entitled "Improved Methods for the Clinical Management of Thalassemia" was issued. Among the suggested areas of investigation were: cardiac and endocrine status after therapeutic use of Desferrioxamine; optimum methods of administering the drug; improved transfusion techniques; and management of endocrine abnormalities.

The major problem areas are: the procurement of sufficient quantities of urinary erythropoietin for further purification and characterization; and the clinical management of thalassemia.

3. Sickle Cell Disease

Efforts directed toward sickle cell disease are an integral part of the overall Division's mission to seek improvement in the diagnosis, prevention, treatment and cure of blood diseases. The program has continued its activities in the development of an effective therapeutic agent, and agents exhibiting anti-sickling continue to be studied. These include carbamyl phosphate, alkylating agents, procaine hydrochloride, cyanate, and zinc. Recent uncontrolled studies have reported that zinc appears to have some effect in reducing the frequency and severity of sickle cell "crises" in sickle cell patients who are zinc deficient.

A major focus is to encourage research on cell mechanisms operating to yield increased amounts of Hb F in erythroid cells of individuals with severe sickle syndromes. The ultimate goal of these findings is to ameliorate the symptomatology of individuals with sickle cell anemia through the ability to maintain production of Hb F that exists in infancy.

Methodologies to identify hemoglobin types in single cells obtained either directly from the fetus or from amniotic fluid during the first trimester of pregnancy continue to be investigated. A method to accurately separate maternal from fetal cells in amniotic fluid is presently under investigation to facilitate prenatal diagnoses. Progress continues to be made in the development of fetoscope with accompanying safe and accurate methods to obtain fetal blood and detect abnormal hemoglobins early in pregnancy.

A workshop on Extracorporeal Techniques was held to explore ways of developing the best mechanism for extracorporeal treatment to maximize the controlled modification of hemoglobin in blood. It was recommended that extracorporeal techniques should be developed to evaluate the therapeutic effectiveness of cyanate and other agents.

The major problem areas identified in this program are: (a) development of effective-non-toxic anti-sickling agents and (b) prevention of overwhelming fatal infection in young infants and children.

4. Blood Resources and Transplantation

The Division has continued its efforts toward the implementation of the National Blood Policy through the support of the American Blood Commission's Task Forces on Regionalization, Commonality, in Blood Banking and National Blood Data Systems. A fourth task force on Donor Motivation has been established and its support is anticipated. A "comparative analysis of regional blood supply systems" was completed which described the dynamics of 12 regional blood supply systems and emphasized their similarities and differences with respect to mode of operation, effectiveness and efficiency. Analysis and design of a national blood resource information system based on standardized reporting, applicable to the blood banking community has been developed in collaboration with the Bureau of Biologics of the Food and Drug Administration. This system (BEIRS) will be implemented as an interim action during 1976.

Hepatitis remains a serious problem in transfused patients. The clinical trials of hepatitis B immune globulin (HBIG) as passive immunization for populations at high risk of contracting hepatitis B are near completion. The Division, in collaboration with the Bureau of Biologics, sponsored a hepatitis workshop to discuss clinical data relevant to the safety and effectiveness of HBIG relevant to licensure. A decision on licensure of HBIG is forthcoming. The incidence of non-A, non-B hepatitis has been identified as a problem of major proportions, more serious than hepatitis A or B. At the present time there is only one study which deals with the total hepatitis problem in transfused blood; i.e., the prospective epidemiological study of post-transfusion hepatitis which is assessing the nature and the extent of the problem. In addition, the advantage of washing frozen thawed red cells in an effort to reduce the incidence of hepatitis is underway in transfused patients. The unavailability of a susceptible animal host to the human hepatitis virus has hitherto hindered progress in this area. The Division is presently supporting a chimpanzee breeding colony in which 20 seronegative champanzees are available for hepatitis research.

A prototype sterile connecting device developed for use as a means of providing a mechanism for sterile multiple entry of blood bags is undergoing clinical trials. Such devices have many applications in blood banking practice, one of which is the extension of the 24-hour outdating period for frozen-thawed red cells.

Considerable progress has been made in the development of synthetic materials for use as artificial blood, however, present materials are still not satisfactory. There is a clear need to develop and test new and improved preparations for use as blood substitutes. The synthesis and biological screening of new fluorocarbon compounds and the test of stroma free hemoglobin for use as artificial blood will soon be initiated. These materials show great promise and could help in solving our collection, storage and hepatitis problems, especially for short periods of time in emergency situations.

Although progress has been made in establishing useful methods for the preparation, storage and transfusion of cellular components of blood, optimal collection, storage and transfusion conditions are yet to be clearly defined. An RFP for the "study of collection, function and transfusion of granulocytes" and an RFA for the preservation of human platelets for transfusion" were recently released.

In the area of transplantation, the Division partially supports and administers the International Bone Marrow Transplant Registry.

A major problem associated with the development of new methods of plasma fractionation and the production of new products is a lack of any sustained commercial research efforts, even though activity in this area shows great promise of providing the health care system with new and better products for therapy. The Division continues to support research attempts to modify established procedures through automation and continuous monitoring. These studies are aimed at increasing the yield and purity of plasma fractions and contribute to a more efficient use of fractions. There are also studies underway to isolate and evaluate new products of plasma fractionation which may be especially useful in deficiency disease.

5. Professional Development and Training

The Division shares the support of training investigators for high quality blood research with the National Institute of Arthritis, Metabolism, and Digestive Diseases and the National Cancer Institute. The Division is supporting over 171 trainees through the National Research Service Awards Individual and Institutional Program and through Research Career Development Awards. The program areas in which training is being provided are thrombosis and coagulation, the blood banking sciences, and red blood cell disorders. A coordinated effort with the Bureau of Biologics is underway to assess the national training needs in the blood banking sciences. Data should be available by November, 1976.

DIVISION OF LUNG DISEASES

ANNUAL REPORT

July 1, 1975 through June 30, 1976

I. MISSION OF DIVISION OF LUNG DISEASES PROGRAMS

The broad program goals of the Division of Lung Diseases are more effective diagnosis, treatment and, ultimately, prevention of pulmonary diseases and respiratory disorders. To these ends the Division emphasizes seven program areas: Structure and Function of the Lung, Emphysema and Chronic Bronchitis, Pediatric Pulmonary Diseases, Fibrotic and Immunologic Lung Diseases, Pulmonary Vascular Diseases, Respiratory Failure, and Prevention, Control and Education. Upon the advice of the Pulmonary Diseases Advisory Committee and other experts, the Division selected these areas after weighing a variety of factors; namely, the magnitude of the health problem of the disease, state of readiness of knowledge and technology for research advances, adequacy of the current research effort, and resources (fiscal and manpower) available to the Division.

In addition to supporting traditional investigator-initiated research, program project and training grants and research career development awards, the Division has stimulated research or training in neglected fields through special goal-oriented programs. Specialized Centers of Research (SCOR) Grants support interdisciplinary research with a clinical emphasis. Research and Demonstration Center Grants include demonstration and education projects addressed to prevention and control of lung diseases. Young Investigator Pulmonary Research Grants encourage--but are not limited to--fundamental investigations. Pulmonary Academic Awards, and Pulmonary Faculty Training Grants and Awards meet special needs relative to pulmonary research and clinical manpower. The Division uses the Research Contract to foster targeted pulmonary research and development projects.

II. PROGRESS TOWARD OBJECTIVES

One of the major obstacles to advances in pulmonary-related research has been the failure of basic scientists to perceive the relevance of their disciplines to lung diseases or to realize that the lung has special features that could contribute new insights to their fundamental investigations. It is just such basic studies that are necessary to understanding the etiology, pathogenesis and natural history of lung diseases, and to improvements in their diagnosis and treatment.

The Division has drawn upon a variety of approaches to help biochemists, immunologists, geneticists and bioengineers to understand lung diseases and to encourage them to work on pulmonary-related problems. By issuing requests for contract proposals (RFPs) and, more recently, requests for grant applications (RFAs), the Division has reached many investigators who have not previously worked on fundamental problems relevant to lung diseases. Studies of isolation, separation and culture of lung cells, initiated in 1973 by a contract

program, have been considerably expanded through investigator-initiated research grants and the SCOR program. A resource facility to characterize, store and distribute individual lung cells has recently been initiated. Other contracts support chemical investigations of elastin and respiratory mucus and immunologic studies of HL-A antigens in relation to lung diseases. Grants that resulted from RFAs are addressed to pathogenesis of inflammation in the lung, and to cultures of lung tissue. Young Investigator Pulmonary Research Grant solicitations have attracted a large number of young researchers who are pursuing fundamental studies relevant to lung diseases. Interdisciplinary research is also supported through 17 Program Project Grants and 16 Pulmonary SCOR Grants. Finally, workshops have provided an ideal medium for exchange of information between fundamental and clinical investigators.

The Division's commitment to a prevention, control and education program is reflected in three contract programs and the recently constituted Task Force on Prevention, Control and Education in Respiratory Diseases. Although this facet of the Division's program has been slow to grow because both the Division and its Advisory Committee have had to learn about the needs and opportunities in this area, the Task Force will develop a program plan that should provide guidance for an effective effort. The Task Force is chaired jointly by Dr. Claude Lenfant, Director of the Division of Lung Diseases, and Dr. Clement R. Brown, Jr., Director of Medical Education at Chicago Community Hospital. It includes not only experts from the academic community of pulmonary physicians and researchers, but also from the broader health professional community concerned with the practical application of biomedical research findings. The Task Force will identify research advances that are potentially applicable to the prevention, diagnosis or treatment of specific respiratory diseases, and are ready to be communicated through demonstration or education projects addressed to practicing physicians, providers of health-related community services, patients or the general public. By December 1976, it will issue a report that will recommend a timely, feasible and manageable program for prevention and control of respiratory diseases through education.

The dearth of well-trained pulmonary clinicians and researchers continues to be a major cause for concern. The Pulmonary Academic Award Program now supports awardees at 39 institutions, an increase of 33 percent since fiscal 1975. In addition, the Division's newest special program--the National Pulmonary Faculty Training Program--supports six centers that are prepared to provide training to applicants sponsored by medical schools wishing to strengthen their pulmonary faculty.

This year, for the first time, the Pulmonary Diseases Advisory Committee undertook--at the request of the NHLI director, Dr. Levy--a critical appraisal of the Division of Lung Diseases National Program, an appraisal addressed to the goals of the program as well as the Division's plans for implementing them. Until now, each year the Committee has addressed itself to particular facets of the total program. It has periodically (in some instances, annually) reviewed the Contract, SCOR and Pulmonary Academic Award programs. This time the review is addressed to substantive program areas; namely Structure and Function of the Lung, Chronic Bronchitis and Emphysema, Pediatric Pulmonary Diseases, Pulmonary Vascular Diseases, Respiratory Failure, Manpower Programs, and Prevention, Control and Education. The Committee's assessment includes

suggestions for the future but is, in general, in accord with the current National Program.

The Pulmonary SCOR Program, usually reviewed annually by the Committee, was not assessed this year because active grants are now competing for renewal and are receiving indepth review by the SCOR Review Panels. However, the Advisory Committee has recommended new evaluative procedures for the future, procedures that will involve outside consultants and will be held 18 and 36 months from initiation of the SCOR. The favorable outcome of the review of SCOR grants that competed for renewal is testimony to the success of this goal-oriented program.

III. HIGHLIGHTS OF ACCOMPLISHMENTS

In a multifaceted program it is difficult to single out specific accomplishments and somewhat unrealistic to attribute achievements to the activities of a particular year; with rare exceptions, such accomplishments are the final expression of work that has been pursued over several years.

Studies of isolated lung cells have progressed to the point that viable, homogeneous preparations are now available for endothelial cells, fibroblasts, macrophages and type II alveolar cells. Moreover, the inclusions in type II cells have been associated with the presence of a surface active material necessary to maintain patency of lung alveoli. The lumenal surface of pulmonary vascular epithelium has been identified as the site of action of enzymes that convert angiotensin I into angiotensin II, a potent, naturally occurring vasopressor.

Elastin, a structural component of the lung that is affected in numerous disease states, has been isolated and chemically characterized. The amino acid composition of lung elastin is essentially the same as aorta elastin from the same species. A soluble precursor (tropoelastin) of lung elastin has been isolated for the first time from rabbit lungs.

Noteworthy advances have been made in purification and analysis of alpha-1-antitrypsin glycoprotein from normal individuals and from individuals homozygous for deficiency of this protease inhibitor. When present in normal amounts, alpha-1-antitrypsin protects against destruction of the lung by proteolytic enzymes from macrophages. However, individuals with genetic deficiency of alpha-1-antitrypsin lack such protection and are predisposed to emphysema. With new improved methods for biochemical analysis, the amino acid and carbohydrate composition of purified preparations has been shown to be different in protease inhibitors from normal individuals and from individuals with alpha-1-antitrypsin deficiency.

Protein components of the surface active material in amniotic fluid have now been purified, enabling investigators to develop specific antibodies that are used to detect minute amounts of the surface active material. This provides a rapid, reliable and inexpensive assay that is a promising tool for prenatal detection of neonatal respiratory distress syndrome. Such early detection is essential to effective intervention in a disorder that is usually fatal unless promptly treated.

The role of prostaglandins in lung metabolism is receiving increasing attention. They seem to have a role in capillary permeability, vascular tone and airway tone and to contribute to homeostatic balance in the pulmonary vasculature. Prostaglandins and related compounds have been isolated from lung perfusates and pulmonary edema foam. They are also released during hypoxia, causing increased vascular resistance and contributing to pulmonary hypertension.

A thoracic window technique has provided a valuable tool for animal studies of the pulmonary microcirculation and the changes that accompany pulmonary embolization.

A roentgenographic technique--microfocal spot magnification--has been developed, a technique that has the power and resolution to enable investigators to visualize pulmonary structures as small as the most peripheral extensions of the airways (respiratory bronchioles). It can be used for cinematographic recording of dynamic events in unfixed preparations of animal and postmortem human lungs. The clinical potential of this technique is quite promising as it utilizes unusually low levels of radiation. By permitting visualization of the lung during tests for pulmonary function, the microfocal spot technique is a promising tool for both the respiratory physiologist and the physician.

Important advances in sensor technology hold great promise for development of blood gas monitors that will provide rapid, accurate assessments of changes in blood gases. If this technology can be coupled with noninvasive methods of monitoring blood gases, it will contribute to early detection and rapid therapeutic intervention for adult respiratory distress dyndrome.

A procedure for auditing medical records, developed under a contract for an education program, will be useful in both the design and assessment of other health education programs. It permits rapid scanning of large numbers of hospital charts and sorts them into various preselected categories. This procedure holds great promise as a way to evaluate changes in practice behavior of health professionals.

IV. REPORTS OF WORKSHOPS, MEETINGS, OTHER

A. Workshop Reports

Lung Cell Metabolism Workshop (Report distributed)
Held February 11-12, 1975 in Airlie, Virginia

Lung Cell Separation, Identification and Culture (Report distributed)
Held March 10-11, 1975 in Airlie, Virginia

Prostaglandins and the Lung (Report in preparation)
Held January 21-22, 1976 in Airlie, Virginia

Isolation, Characterization and Mechanism of Action of Proteases and Antiproteases (Report in preparation) Held February 9-11, 1976 in Airlie, Virginia

B. Other Reports

Division of Lung Diseases Contract Program (Limited distribution) (For fiscal year 1975)

Evaluation of Completed Contract Program: RFP NHLI-71-9: Oxygen Therapy vs. Oxygen Toxicity and Methods for Ventilatory Assist (General Distribution) August 1975

Report to the National Heart and Lung Advisory Council (Limited distribution) September 1975

V. MAJOR PROBLEM AREAS

The Division of Lung Diseases has three major problems:

1) Inadequate Staffing

The problem of inadequate staffing, mentioned in the Division's Annual Reports of 1974 and 1975 (the first years that problem areas were identified in the Annual Report), remains unresolved. In view of expansion of the Division's responsibilities, particularly with regard to prevention, control and education programs, the present ceiling of allowable positions is very inadequate. The problem is two-fold: The number of secretaries is insufficient to serve the needs of the present professional staff. The number of professionals is insufficient to serve the expanding programs of the Division. It is regrettable that this matter has not been corrected despite the recent increase in the Institute ceiling to 747 positions.

2) Inadequate Space

A related problem to that of personnel is the inadequacy of space. This deficiency is so great that a professional staff member is now using an outer office designed originally for a secretary.

3) Grant Assignment

A problem that impedes development of a program consonant with the Division's mandate under Public Law 92-423 is the absence of appropriate procedures for assignment of applications that are submitted in response to initiatives of the Division, such as RFAs and SCORs. Although conceived as part of the Division's National Program, applications in response to such DLD solicitations are frequently assigned to other Institutes. This makes it impossible to develop a forward-looking program that is responsive to national needs and consonant with the Division's mandate.

NATIONAL HEART AND LUNG INSTITUTE
DIVISION OF EXTRAMURAL AFFAIRS
Annual Report
July 1, 1975 - June 30, 1976

The Division of Extramural Affairs is responsible for formulating and coordinating Institute-wide policies for research contracts, grants, and training programs. The Division is the focal point for providing advice to the Director, NHLI, on research contract, grant, and training program policy. It also represents the Institute on overall NIH extramural and collaborative program policy committees, coordinates such policies within NHLI, and coordinates the Institute's research and training program activities with the National Heart and Lung Advisory Council. Other major responsibilities of the Division include: (a) grant and contract management and processing services for the Institute's other divisions, (b) reports and statistics related to the Institute's extramural and intramural programs, and (c) initial scientific and technical merit review of project grants and research contracts for the Institute.

The Division has continued to serve as the primary liaison to the National Heart and Lung Advisory Council, and has continued to develop procedures and mechanisms to facilitate the Council's review of programs and applications. Council members who are primary reviewers for program project applications now give verbal reports when they feel that there is something the Council should take special note of, some special recommendation that should be made by the Council, or when questions are asked by other members of the Council. In reviewing applications to support conferences or meetings, the Council has accepted a set of Staff developed guidelines upon which to base their recommendation. Staff provides an additional statement covering these points when they are not included in the initial review group summary.

The Division continues to provide a broad range of services for the entire Institute. These include:

1. Management functions for grants and contracts.
2. Central storage and maintenance of official files for all grant programs.
3. Preparation of review materials for Council and Staff.
4. Preparation of official and summary minutes of Council actions.
5. Operation of the Policy and Procedures Office.
6. Committee management functions.

The initial technical merit review of research grant applications and research contract proposals has continued to result in markedly increased responsibilities. The types of reviews in the grant program included:

Clinical Trials
Conference Grants
Arterio SCOR Grants
Pulmonary SCOR Grants

Comprehensive Sickle Cell Center Grants
Medical School Pulmonary Training Awards
National Pulmonary Faculty Training Program
Program Project Grants
Pulmonary Academic Awards
Institutional Fellowship Grants
Hypertension in the Young Grants (RFA)
Thrombosis SCOR Grants
Young Pulmonary Investigator Program
Vascular Complications of Diabetes Grants (RFA)

In addition, nineteen contract reviews were held directly related to new RFP's issued by the Institute, and twenty renewal contract reviews were held. Thus, a total of 39 review committees were convened to review approximately 350 contract proposals during Fiscal Year 1976. Furthermore, approximately ten mail reviews, related to unsolicited or sole source proposals, were conducted by the Review Branch.

The Grants Operations Branch was responsible for more than 2,400 awards, with a dollar value in excess of $231,000,000 during Fiscal Year 1976. The Branch's responsibilities included the actual issuance of the awards as well as the day-to-day administration of those awards. The Branch was also actively involved in Council preparation, grant budget negotiations, and award notice preparation, as well as serving as an interdivisional coordinator. The Branch's continued efforts to improve its management practices and procedures have undoubtedly resulted in vast dollar savings for the Institute.

The Contracts Operations Branch was responsible for an expanded contracts program of over 500 contracts representing approximately $100,000,000 during Fiscal Year 1976. The Branch's decentralized system of "co-location" has continued to prove its worth. This Institute was the first to implement a completely decentralized operation in contracting at the NIH, and since then, most other NIH components, with large contract programs, have followed our example. The concept has fostered a close knit partnership between technical and contracts staff, enhancing the entire procurement process. The Branch staff has continued to operate in a highly professional manner despite a rapid and unprecedented growth in the Institute's contracts program.

In the Reports and Evaluation Branch, a major accomplishment has been the development of procedures that allow users of the NHLI Information System to obtain financial reports for grants directly and on demand. There is continuing activity to expand and extend this capability in general, and to include contracts as well as pre-Council data. Progress has been made in improving contract data reporting and new procedures are in the testing stage. An experienced programmer was successfully recruited, and this has permitted the Branch to begin to develop several innovative procedures that will improve the utility of the Financial and Programmatic reporting system. The system was transferred from tape to on-line disc. The master file may now be used with much shorter turn around time. A "commitment file" segment was developed, along with procedures, to provide the following information:

1. Total dollars awarded for active grants with the fiscal year of funding identified, regardless of when funding occurred.

2. Detailed accounting of active projects and those with future commitments. The status of all funding (direct as well as indirect cost) is identified in each instance.

An agreement has been worked out with the Division of Computer Research and Technology, NIH whereby they will assist Branch staff in an in-depth analysis of the Institute's existing Information System, seeking areas which can be further improved. This task has been initiated with a systems analyst from DCRT being on-site full time to work with the Branch staff.

The Freedom of Information Officer for the Institute is located in DEA and continues to received frequent requests (averaging about one per week); some of these have involved eight or more contract or grant files. Some requests are now being answered by program staff (those that involve no denial of information) although records must still be maintained by the Freedom of Information Offices of NHLI and NIH. Implementation of the Privacy Act caused some minor problems, primarily because the persons designated as responsible for the records, and as access officials, are located in DEA, and the Coordinator, through whom all replies must be routed, is in the Office of the Director, NHLI. During much of this year, requests from investigators for copies of their summary statements were handled through both the Freedom of Information and Privacy Act mechanisms, and routed through the Office of Extramural Research and Training, NIH, for clearance before mailing. The majority of Privacy Act requests have been only moderately time-consuming but the number of such requests is increasing.

The problems of inadequate space and strict hiring constraints have continued to plague the Division for much of Fiscal Year 1976. However, near the end of the fiscal year, the Division was provided with much needed additional space and positions, with a strong indication that more would be forthcoming during the "Wedge Period". It is hoped that the new space and positions will enable the Division to keep pace with its ever increasing workload and responsibilities which are a direct and inevitable result of the Institute's expanding grants and contracts programs.

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NATIONAL HEART AND LUNG INSTITUTE

July 1, 1975 - June 30, 1976

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ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMISTRY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

Research in the Section on Enzymes is concerned with studies on biochemical mechanisms of cellular reguation and mechanisms of enzyme action.

(A) Cellular Regulation of Enzyme Activity.

(a) Theoretical Considerations: (i) Cascade systems. A theoretical analysis has been made of three types of regulatory systems which involve cyclic covalent modification of enzymes; namely, (1) monocyclic systems such as occur in the phosphorylation and dephosphorylation of mammaliam pyruvate dehydrogenase, (2) closed bicyclic systems as are involved in the nucleotidylylation-denucleotidylylation of E. coli glutamine synthetase and its regulatory protein, and (3) multicyclic systems typified by the sequential phosphorylation and dephosphorylation of enzymes concerned with activation and inactivation of glycogen phosphorylase. The steady state functions of these cascade systems show that they possess an enormous potential for both positive and negative allosteric control and in addition are capable of a tremendous amplification of primary effector perturbations. They therefore constitute a remarkably flexible regualtory system. By means of allosteric and substrate site interactions, these cascades are capable of sensing fluctuations in the levels of a multiplicity of metabolites and thereby maintain activities that are commensurate with overall cellular demands. Qualitative support for the validity of these theoretical predictions has been obtained from studies with pyruvate dehydrogenase (Reed, et al.,) and with glutamine synthetase (this laboratory) showing that the steady state levels of covalent modification are dependent upon the concentration of multiple metabolites. Projected studies with glutamine synthetase are designed to obtain more rigorous support for the theory.

(ii) Cumulative feedback inhibition. Cumulative feedback inhibition of a regulatory enzyme by multiple inhibitors can occur if there is mutually exclusive binding of the inhibitors at a single site on the enzyme (heterogeneous pool inhibition) or if there is a separate binding site on the enzyme for each inhibitor (independent site inhibition). Results of mathematical and graphical analysis show that kinetic data can be used to differentiate these two mechanisms. A general equation for "hetergeneous pool inhibition" was derived and rate equations for cumulative pool inhibition were derived for competitive, noncompetitive and uncompetitive inhibitors.

(b) Glutamine Synthetase: (i) Feedback Inhibition. Stopped-flow kinetic measurements of changes in glutamine synthetase fluoresence that are associated with the binding of substrates and the feedback inhibitors AMP, alanine, glycine and D-valine, show that there are separate binding sites on the enzyme for each of these effectors and that the inhibitors differ in their capacity to inhibit various steps in the catalytic mechanism

of glutamine synthesis. These results illustrate the need for reassessment of ESR data which showed that alanine and glutamate provoke comparable changes in the enzyme-bound Mn^{2+} resonance spectrum, and led other investigators to conclude erroneously that both ligands bind at the same catalytic site on the enzyme.

In continuing studies on the specificity of feedback inhibition by amino acids, it was found that whereas all D-amino acids inhibit glutamine synthetase, the L-isomers are relatively ineffective except for L-alanine and L-serine and the two direct end products of glutamine metabolism, L-tryptophan and L-histidine.

(ii) Effect of monovalent cations. Monovalent cations differ markedly in their effects on glutamine biosynthetic activity. Whereas K^+, Na^+ and $(CH_3)_2NH_3^+$ stimulate activity at 0.05 - 0.3M, Li^+, Rb^+ and Cs^+ stimulate activity at low concentration ($<$ 0.05M) but inhibit activity at higher concentrations, and triethanolamine and dimethylamine have little or no demonstrable effect. These cation effects are not due to ionic strength nor to shifts in the pH-activity profiles of the enzyme.

(iii) Effect of adenylylation on specific activity and affinity for substrates. The Mg^{2+} supported biosynthetic activity of glutamine synthetase is inversely proportional to the average state of adenylylation. The non- linear relationship between state of adenylylation and specific activity which was noted earlier (see 1969 annual report) probably reflects variations in the effect of adenylylation on the stability of the enzyme to purification procedures. However the further observation that the apparent Km for glutamate in the presence of Mn^{2+} is a nearly linear function of the state of adenylylation supports the earlier conclusion that there are heterologous interactions between adenylylated and unadenylylated subunits within hydrid enzyme molecules.

(iv) Regulation of adenylylation and deadenylylation. Studies of the complex enzyme system that regulates the adenylylation and deadenylylation of E. coli glutamine synthetase have been continuted. To facilitate further purification of the regulatory enzymes, colorimetric procedures have been developed for assay of the adenylyltransferase (ATase), the uridylyl removing enzyme-uridylyltransferase complex (UR-UTase) and the P_{II}-regulatory protein. These procedures are just as sensitive and are more convenient and very much cheaper than earlier radioistopic methods which involved enzyme attachment and detachment of ^{14}C-AMP groups to and from glutamine synthetase.

The UR-UTase complex was further purified and its molecular weight was estimated to be about 140,000. By taking advantage of the fact that the P_{II} protein contains no cysteinyl groups, its purification was greatly facilitated by passage over a mercury-agarose column.

Earlier studies showed that in the presence of uridylylated P_{II}, the ATase catalyzed deadenylylation of glutamine synthetase is stimulated by ATP and α-ketoglutarate and is inhibited by glutamine. A steady state analysis

of these effects indicates that there are separate binding sites on ATase for all three effectors, but whereas the positive action of α-ketoglutarate on the enzyme is antagonized by glutamine, the binding of α-ketoglutarate to the enzyme is enhanced by the binding of ATP, and vice versa.

(v) Regulation of enzyme synthesis. A recent suggestion (La Pointe, et al., J. Bact. 123, 843, 1975) that the level of charged glutamyl tRNA regulates the biosynthesis of glutamate synthase and glutamine synthetase, in E. coli is not readily accommodated by observations in this laboratory showing that glutamine synthetase is maximally derepressed and glutamate synthase is maximally repressed when cells are grown with 60 mM glutamate as the sole nitrogen source, nor by the further observation that cells grown in the presence of cyclic-AMP are derepressed for glutamine synthetase and are repressed for glutamate synthase. Nevertheless, the existence of common control elements in the regulation of the levels of glutamine synthetase, glutamate dehydrogenase and glutamate synthase is suggested by alterations in the patterns of repression and derepression of these enzymes when mutant strains of each and the wild type are exposed to various nutritional conditions.

The possiblity that glutamine synthetase and glutamate synthase share common subunits or possess homologous peptide sequences was contraindicated by the failure of antibodies prepared against either one of the pure enzymes to cross reach with purified forms of the other enzyme. The possibility that glutamate synthase and glutamine synthetase might be associated with one another in vivo is suggested by the observation that the addition of anti-glutamate synthase γ-globulin to crude extracts led to co-precepitation of both enzymes and when added to extracts of a glutamine synthetase deficient strain led to partial reactivation of the glutamine synthetase activity. However, in a reciprocal experiment, addition of anti-glutamine synthetase antibody to crude extracts did not lead to co-precepitation of the two enzymes.

Following up an earlier observation that cyclic-AMP exerts a reciprocal effect on the levels of glutamate synthetase and glutamine synthetase, it was found that antibodies in crude serums prepared against purified cyclic AMP binding protein, CRP, (obtained from W. A. Anderson) cross reacts with purified glutamine synthetase. The anti-CRP γ-globulin does not cross react with purified glutamate synthase nor do anti-glutamine synthetase or anti-glutamate synthase antibodies cross react with CRP.

(c) Regulation of Enzyme Levels. In a continuing effort to develop a model system for studying the regulation of specific enzyme degradation, changes in the concentration of individual enzymes caused by either carbon or nitrogen starvation has been investigated in cultures of E. coli, Klebsiella aerogenes, Saccharomyces cerevisiae and Tetrahymena pyriformis.

(i) E. coli. In E. coli starvation for carbon or nitrogen or deprivation of an essential amino acid leads to an increase in rate of protein degradation as measured by the release of radioactive labelled amino acids from prelabeled intact proteins. Amino acid starvation induces the

"stringent response" which is mediated by guanosine tetra- and penta- phosphates. Studies with wild type E. coli and with relaxed and stringent mutant strains carrying a temperature sensitive valyl-tRNA synthetase clearly implicate the stringent system in at least one aspect of protein degradation. Using a new disc gel electrophoresis technique which permits rapid survey of protein and enzymic activity in crude extracts, the appearance of several new protein bands has been detected in extracts from nitrogen-starved cells. Whether these respresent de novo synthesis of new proteins or the breakdown products of preexisting ones, is under investigation.

(ii) Klebsiella aerogenes. Cell free extracts of K. aerogenes catalyze the inactivation of both endogenous and exogeneous glutamine synthetase. The rate of inactivation and loss of glutamine synthetase antigen increases with pH over the range of 6 to 8 and is inhibited by EDTA. Inactivation is stimulated by Zn^{2+}, Ni^{2+}, or Co^{2+}; Mg^{2+} and Ca^{2+} had no effect. Inactivation of glutamine synthetase by cell free extracts has many features in common with the glutamine degrading system present in chloramphenicol treated intact cells (see last year's report). The cell free extract may therefore provide a good model system to investigate the mechanisms of regulation of specific enzymic degradation.

(iii) Tetrahymena pyriformis. Among several enzymes examined, the activity of phosphofructokinase (PFK) declined precipitously following the transfer of T. pyriformis from rich glucose media to sterile buffer. PFK activity in crude extract is stabilized by 1 mM ATP, is enhanced by 0.1M NaCl and is destabilized by 0.1 MKCl and 1-10 mM $MgCl_2$. These results suggest that the PFK in tetrahymena as in rat liver might be subject to regulation by a phosphorylation-diphosphorylation mechanism. Gel filtration of crude sonic extracts show that PFK as well as pyruvate kinase, and aldolase is associated with a fraction of large particles containing membrane vesicles. The possibility that PFK and other glycolytic enzymes are organized into functional organells on the surface of cellular membranes is being investigated.

(iv) Saccharomyces cerevisiae. During prolonged nitrogen starvation the specific activities of proline oxidase, isocitrate lyase, aspartate kinase, and pyruvate kinase remain constant, the activity of glucose-6-P dehydrogenase increases whereas the activities of TPNH-dependent glutamate dehydrogenase and glutamate-oxalacetate traminase decrease. There is also a striking decrease in the total extractable protein and an increase in the amount of extractable lipids.

(d) Regulation of microtuble assembly. Sodium fluoride and also the microsomal fraction form brain inhibit the assembly of microtubules in crude extracts of brain, but not the assembly of purified brain tubulin. Both inhibitions can be overcome by high levels of GTP. In experiments with isotopically labeled GTP and ATP, it was established that the NaF increases the accumulation of a compound tentatively identified as GMP in crude brain extracts and decreases the conversion of adenine nucleotides to GTP. Subsequent studies showed that GMP inhibits the polymerization of tubulin in crude extracts but has no effect on polymerization of purified tubulin.

Conversely, GDP is a potent inhibitor of purified tubulin polymerization but it supports polymerization of tubilin in crude extracts. The inability of GMP to inhibit polymerization of purified tubulin suggests either the GMP is not directly involved in the control of microtubule assembly or that important protein is lost during purification of the tubulin.

(B) Mechanism of Enzyme Action.

(a) Glutamine synthetase. By means of fast reaction techniques, the rates and amplitudes of changes in the intrinsic fluoresence of glutamine synthetase in the presence of Mg^{2+} were followed during the conversion of ATP, glutamate and NH_3 to glutamine. From the data obtained, the individual rate constants for each of the four sequential reversible steps in catalysis have been evaluated, and the binding constants for all substrates as well as the catalytic constant for the enzyme have been calculated. Excellent agreement between the calculated dissociation and catalytic constants and those determined by a steady state kinetic analysis of the over all reaction support the validity of the catalytic mechanism deduced from the fast reaction experiments.

(b) Leucine 2,3-aminomutase. The vitamin B_{12} dependent mutase that catalyzes the conversion of α-leucine to β-leucine has been further purified from extracts of Clostridium sporogenes and its distribution in other biological material has been determined. Catalysis of the reverse reaction (i.e., β-leucine ⟶ α-leucine) by partially purified preparations is stimulated by a number of cofactors including, FAD, CoA, DPN and pyridoxal phosphate. In addition to several strains of clostridia the enzyme has been detected in rat kidney and in the livers of rat, sheep, Rhesus monkey, African green monkey and in normal human leucocytes and in the liver from a child who died of sepsis secondary to leukemia. Especially noteworthy was the discovery that freshly harvested etiolated bean seedlings contain leucine 2,3-aminomutase activity which is stimulated by coenzyme B_{12}. This activity is inhibited by intrinsic factor and the inhibition can be reversed by the addition of coenzyme B_{12}. This respresents the first example of a vitamin B_{12} dependent enzyme in plants.

Annual Report of the
Section on Intermediary Metabolism
and Bioenergetics
Laboratory of Biochemistry
National Heart and Lung Institute
July 1, 1975 through June 30, 1976

The research activities of the investigators in the Section on Intermediary Metabolism and Bioenergetics have been concerned with (1) The anaerobic metabolism of certain amino acids and nicotinic acid with particular reference to the characterization of the components of the selenium dependent glycine reductase system, the pyruvate-dependent proline reductase and B_{12}-coenzyme-dependent α-methylene glutarate mutase reaction (an intermediate step in the fermentation of nicotinic acid by Clostridium barkeri) and (2) The metabolism of formate and other one-carbon compounds by methane-producing bacteria and characterization of the selenium and molybdenum dependent formate dehydrogenase of Methanococcus vannielii.

Selenium Biochemistry and Anaerobic Oxido-Reduction Reactions:

To date three enzymes have been shown to be selenoproteins. These are avian and mammalian glutathione peroxidase, and the bacterial enzymes, glycine reductase and formate dehydrogenase. The latter two are both produced by the amino acid fermenting organism, Clostridium sticklandii. Clostridial glycine reductase catalyzes the reductive deamination of glycine to acetate and ammonia and forms ATP. One component of this enzyme system is a 12,000 dalton, heat stable selenoprotein that contains one gram atom of selenium per mole. The selenium containing residue has been isolated and shown to be selenocysteine. This is the first essential organoselenium compound in a protein to be identified. There are also two cysteine residues in the protein. The two sulfur amino acids and the selenoamino acid exist in the -SH or -SeH form in vitro only if the protein is kept reduced with borohydride or dithiols. Although the mechanism of the highly specific incorporation of the selenocysteine residue into the protein is not yet known, it is likely to be the result of a post-translational modification. However, no precursor form of the protein has been detected in selenium deficient cells with antibodies produced to the native form of the selenoprotein. Experiments are in progress to see if use of certain protein synthesis inhibitors that effectively prevent growth of C. sticklandii, e.g. chloramphenicol, puromycin, and tetracycline, can provide evidence of such an intermediate. Studies on the structure of the selenoprotein showed the carboxyterminal amino acid to be asparagine and this is preceded by phenylalanine. The NH_2-terminus appears to be blocked. Peptides that contain the selenocysteine moiety were obtained by tryptic digestion of the aminoethylated protein and by cyanogen bromide cleavage. Characterization of these peptides is in progress to obtain information concerning the active site of the selenoprotein.

The formate dehydrogenases of C. sticklandii and of Methanococcus vannielii are both selenium dependent enzymes and share several properties in common; e.g. high temperature optimum (65°C), marked oxygen sensitivity,

inhibition by low levels of cyanide and ability to use tetrazolium as terminal electron acceptor. ^{75}Se-selenocysteine has been isolated from hydrolysates of crude preparations of the enzyme from ^{75}Se-labeled C. sticklandii cells. Pure samples of the ^{75}Se-enzyme from both bacterial sources can be obtained by gel electrophoresis under strictly anaerobic conditions. These will be degraded to establish whether the organoselenium moiety of formate dehydrogenase is actually a selenocysteine residue.

Proline Reductase:

Proline reductase of C. sticklandii and related amino acid fermenting bacteria catalyzes a reductive ring cleavage of proline to δ-amino valeric acid and this serves as an electron sink process. Homogenous preparations of the enzyme from C. sticklandii were shown to consist of 10 similar subunits and each of these contains a covalently-bound pyruvate. Since no free NH_2 terminal groups of the protein were detected, the N-terminal positions of the subunits presumably are blocked by the pyruvate moieties. These pyruvate groups are radioactive and are similar in specific radioactivity to the serine residues of the protein when the organism is cultured in the presence of ^{14}C-serine. It is likely, therefore, that the pyruvate moieties are the result of post-translational modification of N-terminal serine residues.

Of particular interest is the finding that the covalently bound pyruvate cofactor is located on a peptide of about 6000 to 7000 daltons that is released from the protein by mild alkaline hydrolysis. Thus it appears that this pyruvyl-peptide is attached through an ester linkage to the N-terminal position of the larger peptide of each subunit of proline reductase.

Menadione-dependent p-nitrophenyl phosphatase of C. sticklandii:

The amino acid composition of the unusual quinone-dependent phosphatase of C. sticklandii was determined and it was established by two independent methods that there are two half cystine residues in the protein. These presumably are maintained in the reduced form during the catalytic reaction by the added dithiol that is required for enzyme activity. A crude lipid fraction was isolated from C. sticklandii cells that could replace the artificial quinone activator, menadione. Further purification and characterization of the active component should lead to the discovery of the natural quinone cofactor of the phosphatase and provide information concerning its biological role.

α-Methylene Glutarate Mutase:

As part of a continuing investigation on the precise mechanism of the chemical rearrangements catalyzed by various B_{12} coenzyme dependent enzymes, the stereochemistry of the interconversion of α-methylene glutarate and methylitaconate is under study. The reaction is catalyzed by α-methylene glutarate mutase isolated from the nicotinate fermenting organism, Clostridium barkeri. An isomerase catalyzes the next reaction in the sequence, the

conversion of methylitaconate to dimethylmaleate. Since preparations of the B_{12} dependent mutase are difficult to obtain free of the isomerase, the overall rearrangement of ^{14}C and ^{3}H labeled α-methylene glutarate to dimethylmaleate was studied with crude enzyme preparations. Although satisfactory conversion of the ^{14}C labeled substrate to product of the same specific radioactivity was observed, there was substantial loss of tritium from the doubly labeled product. Thus for critical studies on the mechanism of the rearrangement it will be necessary to minimize the loss of tritium in the process. This may be achieved by further purification of the enzyme preparation.

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Annual Report of the
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart and Lung Institute
July 1, 1975 through June 30, 1976

Research in the Section on Protein Chemistry consists of studies on the physical and chemical properties of macromolecules of biological interest and on the roles of ligand binding and protein-protein interactions in enzyme catalysis and regulation.

Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli have been studied by microcalorimetry and equilibrium dialysis techniques. Glutamine synthetase, a strictly regulated enzyme in E. coli, is a dodecamer with twelve catalytic sites. Calorimetrically measured heats have provided information on the separateness of binding sites and have given accurate thermodynamic constants for the binding of low-affinity ligands to the enzyme. A test system for separate ligand sites involved measurements of heats of binding of two substrates, ADP and L-glutamine, to the enzyme; the overall heats of binding for the substrates binding sequentially and simultaneously to the enzyme were equal within experimental error. Using this approach, separate enzyme binding sites were shown for the inhibitors, AMP and L-tryptophan, and for the substrate L-glutamate and the inhibitor L-alanine. Varying the concentration of ligands gives thermal saturation functions which yield accurate affinity constants and enthalpy values for the binding of ligands for which the enzyme has poor affinity. For example, measurements of the binding of glutamine to unadenylylated glutamine synthetase (in buffers at pH 7.1 containing 0.10 M KCl and 0.001 M $MnCl_2$) in the presence and absence of ADP gave K_D' values for glutamine of 1.7 and 7.3 mM and corresponding $\Delta H'$ values of -7.4 and -9.7 kcal/mole, respectively. In addition, using buffers with different heats of protonation quantitates proton uptake or release caused by ligand binding to a protein. In this way, it was found that the binding of AMP to the enzyme causes a net proton uptake of 0.57 equiv of H^+ per mole with $\Delta H' = -10$ kcal/mole after correcting for the proton uptake whereas ADP binding ($\Delta H' = -5.2$ kcal/mole) causes no significant proton uptake or release. This is indirect evidence that the enzyme subunit sites for binding the inhibitor AMP and the substrate ADP are different. In fact, equilibrium binding measurements with [^{14}C]ADP and [^{32}P]AMP have shown directly that both compounds can bind simultaneously to each of the enzyme subunits. However, the rate of AMP binding in the presence of ADP during equilibrium dialysis measurements was extremely slow. Since AMP has been reported to be phosphorylated to ATP by glutamine synthetase under the conditions of the reverse Mn^{2+}-supported biosynthetic reaction, the effects of glutamine, ADP, and inorganic phosphate on the binding of AMP were tested. While inorganic phosphate or glutamine with ADP had little effect on the binding of AMP, the presence of inorganic phosphate with ADP drastically reduced the amount of AMP binding. These and other ligand interactions with glutamine synthetase are being studied further.

Studies on the chemical and regulatory properties of the glutamine synthetase adenylyltransferase from E. coli are continuing. A covalent modification of the allosteric site of this enzyme for glutamine did not occur upon treatment of the adenylyltransferase with an alkylating glutamine analog, 2-amino-4-oxo-5-chloropentanoic acid. However, another glutamine analog, L-2-amino-3-ureidopropionic acid, did activate adenylyltransferase in adenylylating glutamine synthetase.

Studies on the glutamyl-tRNA synthetase (GluRS) of E. coli have indicated that this enzyme is a single polypeptide chain of 63,000 molecular weight. A protease inhibitor (phenylmethylsulfonylfluoride) was included in purification buffers; this reduced the number of active GluRS peaks in hydroxyapatite chromatography from two to one, but increased the amount of protein impurities present. A final purification of GluRS was achieved by preparative gel electrophoresis, during which a protein of ~74,000 molecular weight (74K protein) was separated from the GluRS. This caused a precipitous drop in the specific activity of GluRS which could be partially restored by a readdition of the 74K protein to the GluRS. A possible regulation of the glutamyl-tRNA synthetase by the 74K protein is being investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00201-05 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Metabolism of the Branched-Chain Amino Acids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

J. M. Poston, Ph.D. Section on Enzymes LB NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
National Heart and Lung Institute, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

In the study of the branched-chain amino acids, the initial step in the catabolism of leucine is catalyzed by leucine 2,3-aminomutase. The product, β-leucine, is deaminated and cleaved to give acetate and isobutyrate. Leucine 2,3-aminomutase is dependent upon coenzyme-B_{12} and is stimulated by FAD, coenzyme A, DPN, pyridoxal phosphate. The enzyme is present in clostridia, mammalian liver, human tissue, and plant material. Regardless of the source, the enzyme is linked to cobalamin. A gas chromatographic assay of the branched-chain amino acids has been developed.

PHS-6040
(12-75)

Project Description

Objectives: Pathways for the catabolism of the branched-chain amino acids, leucine, isoleucine, and valine, have been outlined in bovine and rat liver and one or two enzymes have been partially purified and studied, but the catabolism of these amino acids remains incompletely understood. Certain inborn errors of metabolism, e.g. maple syrup urine disease and isovaleric acidemia, have been described which implicate faulty catabolism of these amino acids. The branched-chain amino acids have been shown to participate in the Stickland reaction and many organisms are capable of fermenting these compounds in Stickland pairs. Until recently, a fermentation in which one of these amino acids serves both as the carbon and energy source had not been described. Several strains of organisms have been isolated which grow on leucine in a single amino acid fermentation. The objectives of this project are to establish the fermentation pathways of leucine and the other branched-chain amino acids in these organisms, to examine the enzymes responsible for the various metabolic steps in these fermentations, and to explore the distribution of these pathways in other species.

Major Findings: As reported previously, when cells or extracts of cells of Clostridium sporogenes are incubated with L -leucine, several metabolic products are formed that are consistent with the pathway reported in mammals. However, the production of isobutyrate could not be explained by previously postulated pathways. Evidence was accumulated to support the pathway:

α-leucine → β-leucine → β-ketoisocaproate → acetate + isobutyrate

The first step in this pathway is catalyzed by the enzyme, leucine 2,3-aminomutase. The enzyme, as reported last year, requires coenzyme B_{12} (adenosylcob(III)alamin). In assays utilizing the reverse reaction in which β-leucine is the substrate, production of α-leucine is stimulated by the addition of the cofactors FAD, coenzyme A, DPN, and pyridoxal phosphate. It is not yet clear what part these cofactors play in the migration of the amino group from carbon-3 to carbon-2. Since the reverse reaction has been technically more feasible than the forward reaction, examination of the forward reaction for cofactor requirements has been limited.

Leucine 2,3-aminomutase activity precipitates between 35 and 45 percent ammonium sulfate saturation. Storage of the extracts or fractions in dilute solutions of dithiothreitol has improved recovery of activity. Recovery and storage of activity after passage through DEAE cellulose columns remains poor, however. Purification may depend upon the presence of substrate.

In addition to the finding of leucine 2,3-aminomutase activity in several clostridia, rat, sheep, and Rhesus monkey livers, and rat kidney reported last year, the activity has been found in African green monkey livers and in human tissue. Fresh preparations of normal human leukocytes and liver from a child who died of sepsis secondary to leukemia both showed the presence of the enzyme. From this it is assumed that the pathway exists in these tissues, although the other enzymes have not yet been demonstrated. It was demonstrated

that the normal leukocytes also possess the previously described α-keto pathway of leucine catabolism and that even three-fold excess of β-leucine has no effect on the decarboxylation of carboxyl-labeled α-leucine in the α-keto pathway.

A surprising development was the discovery that freshly harvested etiolated bean seedlings possess leucine 2,3-aminomutase activity. This activity is stimulated by the addition of coenzyme B_{12}. The endogenous activity is inhibited by the addition of intrinsic factor, the mucoprotein that binds cobalamins, and the inhibition can be overcome by the addition of coenzyme B_{12}. This is consistent with the activity found in bacteria and animals and represents the first demonstration of an actual cobalamin-dependent activity in plants.

The assay of α-leucine by ninhydrin is sensitive but slow and not adaptable to numerous measurements if one must use the amino acid analyzer. Ninhydrin measurement of total α-amino acids is rapid but not particularly sensitive nor specific. β-Leucine is not measurable with ninhydrin. Because of these problems it was decided to try gas chromatographic analysis of the branched-chain amino acids. Accordingly, the method of Gehrke using trimethylsilyl derivatives of amino acids was adapted and conditions were established so that all of the branched-chain amino acids can be separated and quantified on columns of OV-11 silicone on Supelcoport. Norleucine can be used as an internal standard. This analytical tool permits measurement of both the forward and reverse reactions.

Proposed Course of Action: The leucine mutase will be purified and characterized. To this end, the conditions which yield maximum activity in cell cultures will be established. The nature of the B_{12} involvement will be established and the nature of the other cofactor involvement in the forward and reverse leucine 2,3-aminomutase reactions will be examined. The distribution of the pathway in mammalian and human tissues will be examined. The β-leucine deaminase (or transaminase) will be examined and the fate of the nitrogen will be determined. The β-ketoisocaproate cleavage enzyme will be examined and its cofactors established.

Relevance to Biomedical Research: This study impinges on at least two areas of medical concern, 1) the mode of action of vitamin B_{12} in its metabolic roles and 2) the means by which organisms catabolize food materals. This second area is directly concerned with several inborn errors of metabolism that have been shown to be devastating to the well-being of humans, especially in the instances of maple syrup urine disease, isovaleric acidemia, and disorders of the catabolism of short-chain acids. The mode of action of B_{12} is imperfectly understood, but its importance in hematopoiesis and in the maintenance of proper neurological function is exemplified in the desease of its metabolis deficiency, pernicious anemia.

Publications:

Poston, J. M.: Leucine 2,3-aminomutase, an enzyme of leucine catabolism. J. Biol. Chem., 251, No. 7, 1859-1863, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00202-05 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Kinetics, Regulations and Mechanisms of Biochemical Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. B. Chock	Section on Enzymes	LB NHLI
	Sue Goo Rhee	Staff Fellow	LB NHLI
	Judith R. Bale	Visiting Scientist	LB NHLI (started 9-5-75)
	E. R. Stadtman	Chief	LB NHLI
	Robert Park	Clinical Associate	LB NHLI
OTHER:	Victoria P. Werth	Summer Fellow	LB NHLI

COOPERATING UNITS (if any) M. Greifner, Biomedical Engineering & Instrumentation Branch Division of Research Service, NIH: S. Chock and E. Eisenberg, Laboratory of Cell Biology, NHLI, NIH; Wolfgang Bergermeister, Laboratory of Chemistry, NIAMDD, NIH

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.1	3.7	.4

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The mechanism of the biosynthetic reaction catalyzed by the Mg^{2+}-supported unadenylylated glutamine synthetase from E. coli has been elucidated to show the complete catalytic cycle. In addition to the enzyme-substrate and enzyme-product complexes, the cycle consists of two fluorescent intermediates. The proposed mechanism is used to explain the observed pH profile and the inhibition exhibited by amino acids. Two amino acid binding sites are proposed, one for L-amino acid and one for D-amino acid; Gly binds to both sites. L-amino acids can bind weakly to the D-amino acid site and vice versa. Binding to the L-amino acid site inhibits the reaction between intermediate 2 and NH_3, whereas the binding to the D-amino acid site will antagonize the binding of glutamate. (2) Theoretical analysis of a regulatory model which involves covalent modification of enzymes shows that a cyclic cascade regulatory system constitutes remarkable potential for both positive and negative allosteric control with tremendous amplification potential. In essence, a controlled cascade system functions like a physiological computer. (3) Other work includes lag-time study, fast kinetics of substrate binding, actomyosin ATPase catalytic cycle, assay methods for adenylylation-deadenylylation, interaction of actin and phalloidin, isolation of ATase, P_{II}, and glutamate synthase, improvement of stopped-flow machine & development of laser heating temperature jump machine.

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PHS-6040
(12-75)

Project Description:

Objectives: (1) To set up a laboratory for the study of fast kinetics of reactions, particularly for studying individual steps of enzymic reactions and protein-ligand interactions. (2) With this fast kinetic technique and other physical and chemical methods, to elucidate the biochemical action of glutamine synthetase and glutamate synthase from Escherichia coli. (3) To analyze theoretically a regulatory model which involves metabolic regulation through a cyclic cascade system for covalent modification of key enzymes. (4) To improve assay methods for the adenylylation, deadenylylation, uridylylation and deuridylylation in the cascade regulatory system for glutamine synthetase and attempt to isolate in large quantity the regulatory enzymes involved for mechanistic study. (5) To study the kinetics and mechanism of DNA--repressor interaction utilizing the fluorescence technique.

Major Development:

(1) We have further developed our present stopped-flow machine to allow simultaneous measurement of both optical density and fluorescence emission changes of a reaction.

(2) A mixing device has been built to allow one to (i) obtain a rapid mixing for slow reactions which can be followed in a spectrophotometer or a fluorometer; (ii) obtain a rapid heat exchange temperature-jump effect such that one can follow a relatively slow reaction which is perturbed by temperature jump.

(3) The neodynium-glass laser has been activated and Q switched such that it is ready for the development of the laser heating temperature-jump machine.

Major Findings:

(1) The mechanism for the biosynthesis of L-glutamine from L-glutamate, NH_3 and ATP, catalyzed by the glutamine synthetase from Escherichia coli has been elucidated further with fast reaction techniques and the intrinsic fluorescence of the protein. When the Mg^{2+}-supported unadenylylated glutamine synthetase was mixed with MgATP in the homemade fast mixing cell, a very fast change in protein fluorescence intensity was observed (half time = 1.4 m sec). This change in fluorescence is caused by the binding of MgATP to $MgE_{1.0}$ (subscript indicates the average number of adenylylated subunits per dodecamer) and the protein conformational change induced by the MgATP binding. However, a relatively slower rate of fluorescence enhancement is obtained when $MgE_{1.0}$-Glu is mixed with MgATP. The analysis of the fluorescence change as a function of time suggests that two or more reaction processes take place. Of the two reactions, the slower reaction is more sensitive to reaction temperature; the faster reaction is a function of MgATP, while the slower reaction is essentially independent of ATP concentration at high ATP concentration. Both the reaction rate and amplitude are the same whether

MgATP is mixed with $MgE\overline{\quad}Glu$ or MgATP plus glutamate is mixed with $MgE\overline{\quad}_{1.0}$. In addition, a similar type of time-course is also observed when glutamate is added to $MgE\overline{\quad}_{1.0}ATP$. Under these conditions, the amplitude observed is only 56% of that observed by adding MgATP to $MgE\overline{\quad}_{1.0}Glu$. The observed time-course suggests a reaction scheme of

$$MgE\overline{\quad}_{1.0}Glu + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MgE\overline{\quad}_{1.0}Glu\ ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} I_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} I_2 \qquad (1)$$

where I_1 and I_2 are reaction intermediates 1 and 2, respectively. The fact that when the order of addition is varied, only amplitudes are different but not the reaction rates observed, indicates that the fluorescence changes induced by I_1 and I_2 formation are very fast relative to the rates of formation of I_1 and I_2. Therefore, the observed time-course reflects the rates of I_1 and I_2 formed. The initial fast reaction rate calculated from the biphasic time-course is essentially the same as the rate obtained when MgATP is added to a mixture of $MgE\overline{\quad}_{1.0}$, glutamate and ammonia. Under these conditions, the slower rate of increasing fluorescence intensity is not observed. This is due to the fact that ammonia reacts rapidly with I_2 and hence the fluorescence intensity reflecting the presence of I_2 is not detected. Based on these facts, the observed rates for the addition of MgATP to a mixture of $MgE\overline{\quad}_{1.0}$, glutamate and ammonia can be attributed to the formation of I_1 and analyzed according to the equation:

$$k_{obs} = \frac{k_2}{1 + (K_1[ATP]_o)^{-1}} + k_{-2} + k_3 \qquad (2)$$

where $K_1 = k_1/k_{-1}$. The above equation is derived with the assumption that $k_{-1} >> k_2$ and $[ATP]_o >> [MgE\overline{\quad}_{1.0}\ Glu{\cdot}NH_3]$. The results of the MgATP dependent study give $K_1 = 3051\ M^{-1}$, $k_2 = 104.1\ sec^{-1}$ and $k_{-2} + k_3 = 44.8\ sec^{-1}$. The value of $k_2 + k_{-2} + k_3$ can also be obtained by a different order of substrate addition and the result so obtained is in relatively good agreement with that calculated above.

With the assumption that the total fluorescence change observed upon addition of MgATP to $MgE\overline{\quad}Glu$ is due to the sum of the fluorescence levels of intermediates 1 and 2, it is possible to evaluate all the rate constants in reaction (1). Utilizing the values of k_2, $k_{-2} + k_3$, K_1, and equations 3 and 4, we have evaluated k_{-2}, k_3 and k_{-3} by computer iteration method.

$$\text{Amp } 1 = 1.15\ (k_2 + k_{-2})\ \left[\frac{k_{-3}}{\alpha_1\alpha_2} + \frac{k_{-3} - \alpha_1}{\alpha_1\alpha_2(\alpha_1 - \alpha_2)} e^{-\alpha_1 t} + \frac{k_{-3} - \alpha_2}{\alpha_2(\alpha_2 - \alpha_1)} e^{-\alpha_2 t}\right] \qquad (3)$$

$$\text{Amp } 2 = [2.36\,\alpha_1\alpha_2 - 1.15\,k_{-3}\,(k_2 + k_{-2})]\left[\frac{1}{\alpha_1\alpha_2} + \frac{1}{\alpha_1(\alpha_1 - \alpha_2)}\,e^{-\alpha_1 t} + \frac{1}{\alpha_2(\alpha_2 - \alpha_1)}\,e^{-\alpha_2 t}\right] \quad (4)$$

where Amp 1 and Amp 2 are expected amplitudes due to the formation of I_1 and I_2 respectively; the values of 1.15 and 2.36 are relative fluorescence intensity observed experimentally, and

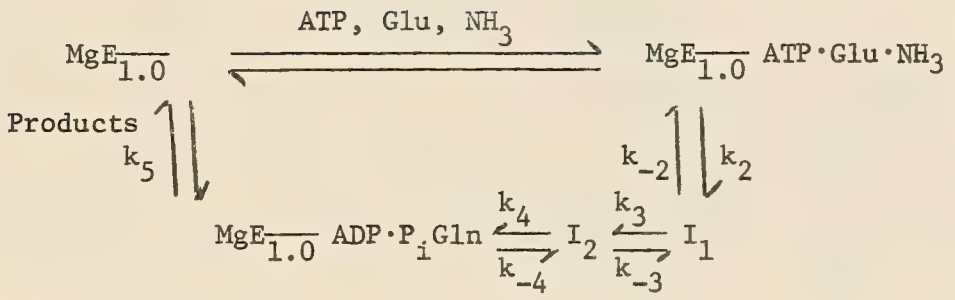
$$\alpha_{1,2} = \frac{(k_2 + k_3 + k_{-2} + k_{-3}) \pm \sqrt{(k_{-2} - k_3 + k_{-2} - k_{-3})^2 + 4k_{-2}k_3}}{2}$$

The rate constants so evaluated are $k_2 = 104.1$, $k_{-2} = 28.8$, $k_3 = 16$, and $k_{-3} = 7.06$ sec^{-1} at 15°, pH 7.0.

The rate constants k_4 and k_{-4} (see scheme 1) can be evaluated by reacting NH_3 with $MgE\underline{\quad}GluATP$ in the stopped-flow machine. It is important to carry out this reaction before a significant amount of ATP is hydrolyzed due to the ATPase activity of the enzyme. The results are best fit with a mechanism shown in equation (5).

$$I_2 + NH_3 \underset{}{\overset{K_a^{NH_3}}{\rightleftharpoons}} I_2NH_3 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} MgE\underline{\quad}ADP\cdot P_i Gln \quad (5)$$

The constants so calculated are $K_a^{NH_3} = 1198\ M^{-1}$ and $k_4 = 97.8$, $k_{-4} = 36.2$ sec^{-1}. With these constants, K_d for NH_3 can be calculated to be 0.31 mM which is in good agreement with the K_m value obtained for NH_3 in the biosynthetic reaction (K_m = 0.3 mM). If a limited amount of NH_3 was added with $MgE\underline{\quad}Glu$ to an excess of MgATP solution, a rapid increase in fluorescence intensity due to formation of I_1 was observed, and the fluorescence intensity remained essentially constant till the NH_3 was consumed; at this time a new fluorescence level, which is corresponding to I_2, was established. From the time required to shift the fluorescence intensity from I_1 to I_2, it is possible to evaluate k_{cat}, which is 6.5 sec^{-1} at 15°C pH 7.0. The data obtained here and those reported previously [Rhee, Chock and Stadtman, Biochimie, 1976; Timmons, Rhee, Luterman and Chock, Biochem. 13, 4479 (1974); Rhee and Chock, Biochem. 15 (1976)] suggest that the catalytic cycle of glutamine biosynthesis when catalyzed by the Mg^{2+}-supported unadenylylated enzyme can be described as:



Scheme I.

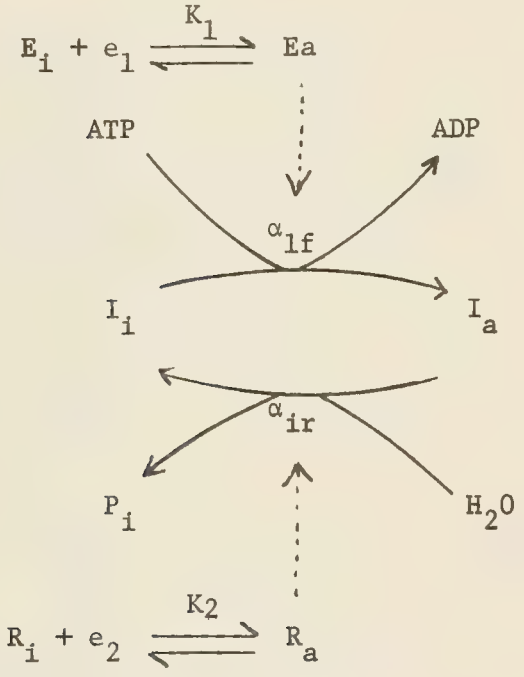
It is assumed that substrates bind randomly and rapidly and that the release of products is also a rapid step (Rhee and Chock, Biochem. 15, 1976). The assumption of a random substrate addition mechanism as shown in Scheme I is based on the facts that: (i) there are synergistic effects observed in the binding of ATP and glutamate as disclosed by fluorescence titrations [Timmons, Rhee, Luterman and Chock, Biochem. 13, 4479 (1974)]. (ii) The total fluorescence enhancement due to ATP binding can be obtained when ATP is added to $MgE_{1.0}$—Glu or Glu is added to $MgE_{1.0}$—ATP; this indicates that glutamate can bind to the enzyme prior to ATP. (iii) NH_3 can bind to $MgE_{1.0}$—ATP and produce a stronger fluorescence enhancement for L-alanine binding. (iv) Fluorescence enhancement is observed when NH_3 is bound to $CaE_{1.0}$—Glu. With the evaluated rate constants and the value of 100 sec^{-1} for k_5 calculated from ADP binding study, one can calculate k_{cat} with equation (6) to be 8.2 sec^{-1}.

$$k_{cat} = \frac{k_2k_3k_4k_5}{(k_2k_3 + k_2k_{-3} + k_{-2}k_{-3})(k_{-4}+k_5) + k_4(k_2k_3 + k_2k_5 + k_3k_5 + k_{-2}k_5)} \qquad (6)$$

This is in reasonable agreement with the values of 7.2 sec^{-1} obtained from steady-state assay conditions and 6.2 sec^{-1} from the fluorescence stopped-flow experiment. In essence, a catalytic cycle for the biosynthesis of glutamine is demonstrated. When the catalytic cycle was studied with varying pH from 6.0 to 8.0, the results show that the rates of both the formation of I_2 from I_1 and the NH_3 attacking of I_2 increase nonlinearly with an increasing pH; the rate for I_1 formation also varies with the pH, but the response is not as pronounced. Analysis of these rates suggests that an amino acid residue with a pK_a of about 7.5 plays an important role in enzymic activity. The reaction amplitudes due to I_1 and I_2 formation and the NH_3 attacking step also exhibit pH dependency. The amplitude data suggest that an amino acid residue with a pK_a of about 6.3 is involved in the catalysis. In addition, at high pH, 7.2 and above, the fluorescence signal indicates a formation of an inactive species in the presence of glutamate and ATP. The equilibrium is shifted toward this inactive species as one increases the pH. Analysis of the pH data based on Scheme I and the formation of the observed inactive species gives a qualitative agreement with the pH profile observed for the biosynthetic activity in the steady-state assay.

(2) Theoretical analysis of a regulatory model which involves cyclic covalent modification of enzymes shows that such a regulatory system constitutes a remarkably enormous potential for both positive and negative allosteric control and a tremendous amplification potential. This model is patterned after (i) the cascade regulatory system of glutamine synthetase in Escherichia coli which involves nucleotidylylation-denucleotidylylation of the regulatory protein, P_{II}, and glutamine synthetase; (ii) the cascade which regulates the phosphorylase and glycogen synthetase in which a series of protein kinases and phosphatases catalyze the phosphorylation and dephosphorylation of the target enzymes; (iii) phosphorylation-dephosphorylation of animal pyruvate dehydrogenase complex, hormone-sensitive lipase and acetyl CoA carboxylase.

Scheme II depicts a typical monocyclic system, where



Scheme II.

E_i and E_a are inactive and active enzyme catalyzing the covalent modification reaction, respectively, I_i and I_a are inactive and active interconvertable enzyme, respectively; R_i and R_a are inactive and active enzyme catalyzing the demodification reaction, respectively; e_1 and e_2 are allosteric effector 1 and 2, respectively; $\alpha_{1f} = \frac{k_{1f}}{K_{1f}}$ and $\alpha_{1r} = \frac{k_{1r}}{K_{1r}}$. k_{1f} and k_{1r} represent the catalytic rate constants for the forward and reverse reaction, respectively; K_{1f} and K_{1r} are dissociation constants for E_aI_i and R_aI_a complex, respectively. K_1 and K_2 are dissociation constants for the activation of E_i and R_i, respectively. In order to simplify the mathematical expressions, we assume: (i) The ATP dependence in the interconversion of the enzymic reaction

is ignored since the concentration of ATP *in vivo* is maintained at a nearly constant level which is several orders of magnitude higher than the substrate enzymes. (ii) The formation of enzyme-enzyme complexes proceeds via a rapid equilibrium mechanism. (iii) The concentration of the enzyme-enzyme complex is negligibly small such that $[I] \simeq [I_i] + [I_a]$; $[E] \simeq [E_i] + [E_a]$; $[R] \simeq [R_i] + [R_a]$, where [I], [E] and [R] are total concentration of I, E and R respectively. With these assumptions, the fraction of I in activated form at time t can be calculated by equation (7) and under steady state equilibrium conditions, this fraction is expressed by equation (8).

$$\frac{[I_a]}{[I]} = \frac{\alpha_{1f}[E_a]}{\alpha_{1f}[E_a] + \alpha_{1r}[R_a]} \left(1-e^{-(\alpha_{1f}[E_a] + \alpha_{1r}[R_a])t}\right) \tag{7}$$

$$\frac{[I_a]}{[I]} = \frac{\alpha_{1f}[E_a]}{\alpha_{1f}[E_a] + \alpha_{1r}[R_a]} \tag{8}$$

where $[E_a] = \frac{[E][e_1]}{[e_1] + K_1}$, $[R_a] = \frac{[R][e_2]}{[e_2] + K_2}$

Equations (7) and (8) indicate that both the rate and the steady level of $\frac{[I_a]}{[I]}$ are functions of 6 parameters which can be varied independently or simultaneously by the results of additional interactions between allosteric effectors and the enzymes involved. It is evident that such a cyclic system is remarkably flexible in its regulatory properties. The alternative mechanisms with respect to the role of e_1 and e_2 in the monocyclic system are: (i) e_2 inactivates R while e_1 activates E; (ii) e_1 activates E and inactivates R; and (iii) e_1 activates both E and R.

In the regulation of glutamine synthetase from *E. coli*, a "closed" bicyclic cascade system is used. The term "closed" is being introduced because both modified and unmodified forms of one of the interconvertible enzymes are catalytically active and operate in an opposite manner, i.e. P_{II} is involved in the adenylylation while $P_{II}(UMP)_4$ is involved in deadenylylation of glutamine synthetase. In this case, no analytical expression can be obtained for the differential rate equations, but the steady state expression can be derived easily. For example, in the regulation of glutamine synthetase, one cycle involves the uridylylation and deuridylylation of the regulatory protein P_{II}, and the other involves adenylylation and deadenylylation of glutamine synthetase. Under most physiological conditions, adenylylated glutamine synthetase is inactive, therefore the enzymic activity can be expressed by the state of adenylylation in the dodecamer. With the assumption that ATase is in large excess relative to the concentration of P_{II} and $P_{II}(UMP)_4$, the state of adenylylation is determined by the expression:

$$\overline{GS}_n = \frac{12\,\alpha_1\alpha_3[UR]}{\alpha_2\alpha_4[UT] + \alpha_1\alpha_3[UR]} \qquad (9)$$

in which $\overline{n}$ is the average number of covalently bound adenyl groups per dodecamer, α_1, α_2, α_3, and α_4 are catalytic constants for the deuridylylation, uridylylation, adenylylation and deadenylylation reaction respectively. The catalytic constant, α, is defined as a ratio of k_i to K_i, where k_i is catalytic rate constant and K_i is the dissociation constant for an enzyme complex which consists of the enzyme being modified and the catalyzing enzyme. UR and UT are uridylyl removing enzyme and UTase respectively. The 10 parameters involved in equation (9) can be varied independently or simultaneously in response to the fluctuations in the concentrations of different metabolites, such as α-ketoglutarate, glutamine UTP, CMP, ATP, etc.

In the case of a multicyclic system, at steady-state equilibrium, the fraction of the target enzyme which is in active forms is:

$$\frac{[I_{na}]}{[I_n]} = \left[\left(\frac{K_d}{[e]} + 1\right)\frac{[R_1][R_2]\cdots[R_n](\alpha_{1r}\alpha_{2r}\cdots\alpha_{nr})}{[E][I_1][I_2]\cdots[I_{(n-1)}](\alpha_{1f}\alpha_{2f}\cdots\alpha_{nf})} + \frac{[R_2][R_3]\cdots[R_n](\alpha_{2r}\alpha_{3r}\cdots\alpha_{nr})}{[I_1][I_2]\cdots[I_{(n-1)}](\alpha_{2f}\alpha_{2f}\cdots\alpha_{nf})} + \cdots + \frac{[R_n]\,\alpha_{nr}}{[I_{(n-1)}]\,\alpha_{nf}} + 1\right]^{-1} \qquad (10)$$

Equation (10) indicates the tremendous amplification potential of such cascade systems. For example, the concentration of e required to produce 50% conversion of I_n to I_{na}, decreases in such a manner that log $[e_{0.5}]$ is inversely proportional to the number of cycles when

$$\frac{\alpha_{1r}[R_1]}{\alpha_{1f}[E]} = \frac{\alpha_{ir}[R_i]}{\alpha_{if}[I_i]} = 0.1.$$

In addition to the controlled amplification capacity, the enormous potential for allosteric control is obvious.

In essence, controlled cascade systems(the uncontrolled system is the unidirectional cascade) function like physiological computers. Their circuitry consists of a series of interconnected terminals in the form of interconvertible enzymes. By means of allosteric and active site interactions, these interconvertible enzymes are programmed to sense fluctuations in the concentrations of a multiplicity of metabolites. This leads to automatic adjustments in the specific activities and rate constants of the several cascade enzymes. Through this system, the multiple inputs are integrated and registered as a single output, namely the fractional modification of the target enzyme which ultimately determines its catalytic activity.

(3) Results of mathematical and graphical analysis of "pool cumulative" inhibitors (all inhibitors bind at the same site) and cumulative inhibitors

(all inhibitors bind at a different site) indicate that kinetic data of a multi-inhibitor study can be used to differentiate the mechanism of inhibition by a given inhibitor with respect to both substrate and other inhibitor(s) presence. A general equation for all "pool cumulative" inhibition was derived. In addition, rate equations for cumulative inhibition were derived for competitive, noncompetitive and uncompetitive inhibitors.

(4) Utilizing the lag-time observed with coupled assay, we have demonstrated that the rate-limiting step for activating divalent-metal-free glutamine synthetase (relaxed enzyme) is a slow conformational change step induced by Mg^{2+} (or Mn^{2+}) binding to the relaxed enzyme. With a high concentration of the coupled enzymes, pyruvate kinase and lactate dehydrogenase, and with fully active glutamine synthetase, the lag-time caused by the coupled enzymes can be made negligibly small. Under these conditions, when relaxed enzyme is used, the rate of absorbance change at 340 nm due to the conversion of DPNH to DPN^+, can be expressed as:

$$R = v\left[t - \frac{1}{k_a}\left(1 - e^{-k_a t}\right)\right] \qquad (11)$$

where R is the absorbance at 340 nm at time t, v is the catalytic rate observed when the total glutamine synthetase is fully active and k_a is the rate constant for the rate binding step in converting the relaxed enzyme to active enzyme.

Experimental results under the conditions described above show that no lag-time is detected when $MgE\overline{}_{1.0}$ is used. When the relaxed enzyme is used, with or without preincubation with ATP, NH_3, or ATP-NH_3 mixture, a lag-time which corresponds to a k_a value of 0.012 sec^{-1} is observed. The calculated k_a is in good agreement with the first order rate constant (0.011 sec^{-1}) obtained from the rate of absorbance change at 290.3 nm induced by Mg^{2+} or Mn^{2+} binding to the relaxed enzyme (also see Hunt and Ginsburg, Biochem. 11, 3723, 1972). Relaxed enzyme plus glutamate exhibits the same lag-time if the mixture is assayed after 10 min. incubation at 0°C; but a shorter lag-time is observed when the mixture is incubated at 30°C for 30 min. or longer. This suggests that glutamate binds the relaxed enzyme in such a manner that the $E\overline{}_{1.0}Glu$ has a larger k_a than $E\overline{}_{1.0}$ or the relaxed enzyme is in equilibrium with $E\overline{}_{1.0}Glu$ and $E\overline{}_{1.0}Glu$ is active. When relaxed enzyme is incubated with high Ca^{2+} concentration (3.3 mM), no lag-time is observed; and the lag-time observed with low Ca^{2+} concentration can be shortened by adding glutamate. This suggests a synergistic effect between Ca^{2+} and glutamate binding to the relaxed enzyme. The fact that $CaE\overline{}_{1.0}$, $CaE\overline{}_{1.0}Glu$ and $MgE\overline{}_{1.0}$ do not exhibit the same difference spectra in the UV region, and yet they are capable of activating the relaxed enzyme indicates that (i) the same difference spectra are not required for converting the relaxed enzyme to an active enzyme. (ii) $CaE\overline{}_{1.0}$ is capable of supporting the biosynthetic activity if MgATP is used as a substrate. The possibility that $CaE\overline{}_{1.0}$ exists in a unique conformation such that Mg^{2+} can rapidly activate the enzyme without causing a lag-time is unlikely because further addition of Mg^{2+} or Mn^{2+} to the $CaE\overline{}_{1.0}$ produces an additional absorbance change at 290.3 nm with a relatively slow rate. In essence, we have demon-

strated: (i) The rate for activating a relaxed enzyme by Mg^{2+} is the same as the rate observed for Mg^{2+} or Mn^{2+} induced absorbance change at 290.3 nm. (ii) Difference spectra obtained between relaxed enzyme and $CaE_{1.0}$, $MnE_{1.0}$ or $CaE_{1.0}Glu$ are distinctly different, yet the Ca^{2+}, Mn^{2+} and Ca^{2+}-Glu mixture are capable of activating the relaxed enzyme. This demonstrates that not all the protein conformational changes induced by Mn^{2+}, Ca^{2+} or a Ca^{2+}-Glu mixture are necessary for activating the relaxed enzyme. (iii) Glu (incubated for 10 min. at 0°C), NH_3, ATP or NH_3 ATP mixture do not affect the rate of activation when incubated with the relaxed enzyme. (iv) Glu and Ca^{2+} exhibit a synergistic effect when they are used to activate the relaxed enzyme.

(5) Unlike the Mg^{2+} or Mn^{2+} supported unadenylylated glutamine synthetase, the protein intrinsic fluorescence is enhanced when glutamate binds to $CaE_{1.0}$ or $CaE_{1.0}NH_3$ and when NH_3 binds to $CaE_{1.0}Glu$. This demonstrates that NH_3 can bind to glutamine synthetase in the absence of ATP and prior to the formation of the reactive intermediates from ATP and glutamate, With these fluorescence intensity changes and stopped-flow techniques, the kinetics of glutamate, NH_3 or ATP binding to $CAE_{1.0}$ were studied at pH 7.0 and 10°C. Analysis of the data indicates that a three-step reaction mechanism is the best fit for the glutamate binding to $CaE_{1.0}$ or $CaE_{1.0}NH_3$, while a two-step mechanism is consistent for NH_3 or ATP binding to $CaE_{1.0}Glu$. In both mechanisms (equation (12)), the substrate first binds to the enzyme in a rapid equilibrium

$$E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES \underset{K_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES'' \qquad (12)$$

manner, followed by substrate induced protein conformational change step(s). The values of K_1 calculated are 60 ± 22 M^{-1}, 16 ± 4.8 M^{-1}, 101 ± 30 M^{-1} and (6.5 ± 0.8) x 10^3 M^{-1} for the reaction of Glu + $CaE_{1.0}$, NH_3 + $CaE_{1.0}Glu$, Glu + $CaE_{1.0}NH_3$ and CaATP + $CaE_{1.0}Glu$, respectively. The $\Delta G°$ for $ES \rightleftharpoons ES'$ step is -0.8, -1, -1 and -2.5 kcal mole $^{-1}$ for Glu + $CaE_{1.0}$, NH_3 + $CaE_{1.0}Glu$, Glu + $CaE_{1.0}NH_3$ and CaATP + $CaE_{1.0}Glu$, respectively. Rate constants, k_2 and k_{-2} for the isomerization step are 171 ± 12 and 40 ± 9 sec^{-1} for Glu + $CaE_{1.0}$; 200 ± 14 and 34 ± 13 sec^{-1} for NH_3 + $CaE_{1.0}Glu$, 122 ± 20 and 21 ± 8 sec^{-1} for Glu + $CaE_{1.0}NH_3$; and 160 ± 6 and 2 ± 1.5 sec^{-1} for CaATP + $CaE_{1.0}Glu$. When second isomerization step is involved, the value of k_3 + k_{-3} is (6.3 ± 1.2) x 10^{-4} and (6.8 ± 1.4) x 10^{-4} sec^{-1} for Glu binding to $CaE_{1.0}$ and $CaE_{1.0}NH_3$ respectively.

The dissociation constant for Glu from $CaE_{1.0}$ is 3.2 mM and Glu from $CaE_{1.0}ATP$ is 0.55 mM. There is a five fold decrease in the presence of ATP. Since ATP can bind independently to $CaE_{1.0}$ as indicated by the enhancement of the protein fluorescence, the data indicate a mutual enhancement between ATP and Glu in binding to $CaE_{1.0}$. With a dissociation constant of 2 μm for ATP from $CaE_{1.0}Glu$ and a mutual enhancement reaction scheme, the value of K_d can be calculated to be 12 μM for ATP from $CaE_{1.0}$. This type of mutual enhancement between ATP and Glu on binding to the enzyme is also observed

for the Mg^{2+} supported enzyme (Timmons, Rhee, Luterman and Chock, Biochem. 13, 4479, 1974). In the Mg^{2+} system, two reactive intermediates are formed upon addition of Glu and ATP to the enzyme (Rhee and Chock, Proc. Nat. Acad. Scie. USA 73, 476, 1976). However, only one exponential function is observed for CaATP + $CaE\underset{1.0}{\text{---}}$Glu. This suggests that in the Ca^{2+} system intermediate 2 (I_2 of Scheme I) is not formed. If NH_3 reacts only with intermediate 2 to form glutamine, then no biosynthesis of glutamine is expected with the Ca^{2+} system. However, as shown in the lag-time study, in the presence of MgATP, $CaE\underset{1.0}{\text{---}}$ is capable of supporting biosynthetic activity.

(6) The kinetics of the protein fluorescence change exhibited by ADP or orthophosphate addition to the Mg^{2+} or Mn^{2+} activated unadenylylated glutamine synthetase were studied by stopped-flow measurements. The kinetic patterns of these reactions are incompatible with a simple bimolecular binding process and a mechanism which required protein isomerization prior to substrate binding. They are consistent with a mechanism in which direct substrate binding is followed by a substrate -induced conformational change step, $ES \rightleftharpoons ES^*$. At pH 7.0 and 15°C, the association constants for the direct binding (K_1 as in equation 12) of ADP to $MnE\underset{1.0}{\text{---}}$ and of orthophosphate to $MnE\underset{1.0}{\text{---}}$ADP are 3.9×10^4 and $2.28 \times 10^2 M^{-1}$, respectively. The association constant for the direct binding of ADP to $MnE\underset{1.0}{\text{---}}P_i$ is $2.3 \times 10^4 M^{-1}$ at pH 7.0 and 9°C. The $\Delta G°$ for the substrate-induced conformational step are -3.5 and -1.3 kcal $mole^{-1}$ due to ADP binding to $MnE\underset{1.0}{\text{---}}P_i$ and $MnE\underset{1.0}{\text{---}}$, respectively, and -1.4 kcal $mole^{-1}$ due to orthophosphate binding to $MnE\underset{1.0}{\text{---}}$ADP. Rate constants, k_2 and k_{-2} (see equation 12) for the isomerization step are 90 and 9.5 sec^{-1} for ADP binding to $MnE\underset{1.0}{\text{---}}$, 440 and 0.36 sec^{-1} for ADP binding to $MnE\underset{1.0}{\text{---}}P_i$, and 216 and 1.8 sec^{-1} for orthophosphate binding to $MnE\underset{1.0}{\text{---}}$ADP. It is worth pointing out that the strong synergism exhibited between ADP and orthophosphate binding to the $MnE\underset{1.0}{\text{---}}$ (Timmons, Huang, Stadtman and Chock, in "Metabolic Interconversion of Enzyme" Fisher, Krebs, Neurath and Stadtman Ed. 1974, 209-220; Hunt, Smyrniotis, Ginsburg and Stadtman, Arch. Biochem. Biophys. 166, 102-124, 1975) is derived from the ADP induced protein conformational change step where the presence of Pi increases k_2 and decreases k_{-2}. Due to the low substrate affinity, the association constant for direct orthophosphate binding to $MnE\underset{1.0}{\text{---}}$ was estimated to be approximately 230 M^{-1} and k_2 = 750 sec^{-1}, k_{-2} = 250 sec^{-1}. At 9°C the estimated association constants for the direct ADP binding to $MgE\underset{1.0}{\text{---}}$ and $MgE\underset{1.0}{\text{---}}P_i$ are 1.8×10^4 and $1.6 \times 10^4 M^{-1}$, respectively; and the rate constants for the isomerization step associated with the corresponding reaction are k_2 = 550 sec^{-1}, k_{-2} = 500 sec^{-1}, and k_2 = 210 sec^{-1}, k_{-2} = 100 sec^{-1}. From the kinetic analysis it is evident that the inability of Mn^{2+} to support biosynthetic activity of the unadenylylated enzyme is due to the slow rate of ADP release from the $MnE\underset{1.0}{\text{---}}ADP \cdot P_i$ complex. In contrast the large k_{-2} obtained for ADP release from the $MgE\underset{1.0}{\text{---}}$ADP or $MgE\underset{1.0}{\text{---}}ADP \cdot P_i$ complex indicates that the release of ADP is not rate limiting in the biosynthesis of glutamine, since k_{cat} obtained under the same conditions is 7.2 sec^{-1}. In addition, the value of k_{-2} (equivalent to k_5 in Scheme I) when used to calculate k_{cat} in Scheme I, gives a reasonable value for k_{cat} [See (1)].

(7) Fast reaction kinetic studies of the interaction of actin, subfrag-

ment-one of myosin and ATP were carried out with the collaboration of Drs. Stephen Chock and Evan Eisenberg. In these studies, light scattering was used to monitor the interaction of subfragment-one with actin and protein. Intrinsic fluorescence was used to detect the formation of intermediates. A single cycle of ATP hydrolysis by a complex of actin and myosin subfragment-one was investigated. The results show that the addition of a stoichiometric amount of ATP to subfragment-one-actin complex causes a cycle consisting first of a rapid dissociation of the subfragment-one from actin by ATP; second, a slower fluorescence change in subfragment-one which may be related to the initial phosphate burst; and third, a much slower rate limiting recombination of the subfragment-one with actin, as indicated by the rerise of turbidity. The rate of this latter step is equal to the actin-subfagment-one steady-state ATPase rate at both low and high actin concentrations, and like the steady-state ATPase, it exhibits a V_{max} of 0.9 sec^{-1} at high actin concentration. Analysis of the data indicate that the release of ADP and orthophosphate is not the rate limiting step in the actin-subfragment-one ATPase; instead, a slow first-order step corresponding to the previously postulated transition from the refractory to the non-refractory state precedes the rebinding of the subfragment-one to the actin during each cycle of ATP hydrolysis.

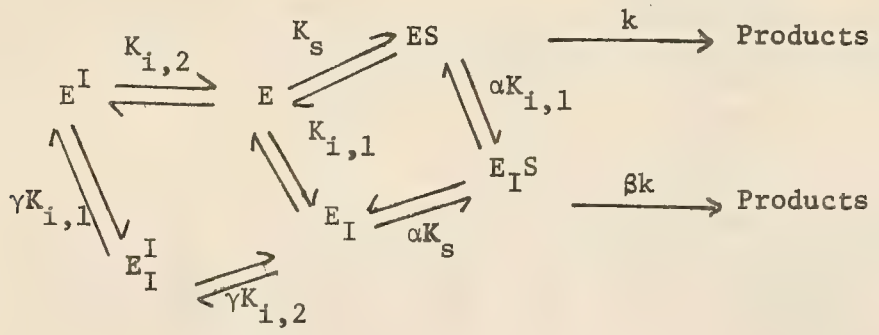
(8) Development and modification of enzymic assay: (a) The reverse biosynthetic reaction catalyzed by unadenylylated glutamine synthetase is measured by a coupled assay method. The coupled enzymes used are hexokinase, glucose-6-phosphate dehydrogenase and diaphorase. The overall reaction is being monitored by the formation of the reduced iodo-nitro-tetrazolium violet at 500 nm. The non-linearity of this assay is caused by the insolubility of the reduced dye, and it is overcome by adding a surfactant to the assay mix. The advantages of this assay are: (i) it provides a higher sensitivity since the extinction coefficient of the reduced dye at 500 nm is three times higher than NADH at 340 nm. (ii) it allows one to detect the enzymic activity qualitatively visually and quantitatively by a cheap visible spectrophotometer. (b) Colorimetric assays for ATase, P_{IIA} and P_{IID}, the enzyme and regulatory proteins which catalyzed the adenylylation and deadenylylation of glutamine synthetase, have been modified to the extent that reproducibility and linearity with respect to the enzymic activity are established. The enzymic activity is monitored by a modified γ-glutamyl transfer reaction which allows one to detect the activity of the unadenylylated subunits and the adenylylated subunits at a relatively high glutamine synthetase concentration. When adenylylation reaction is being studied, alanine is used to suppress the activity of the unadenylylated subunits such that a lower background can be achieved. These assays are sufficiently sensitive and accurate to be used on either crude extracts or pure proteins. In addition, slight modification of these assays will allow one to monitor the activity of UTase and uridylyl removing enzyme.

(9) Large scale purification of glutamate synthase from *E. coli*, ATase and P_{II} from both *Pseudomonas putida* and *E. coli* are in progress. Glutamate synthase was isolated using the procedure of Miller and Stadtman (JBC 247, 7407, 1972) with an additional step: reverse ammonium sulfate chromatography in which glutamate synthase was added to DEAE cellulose in 50% satu-

rated ammonium sulfate and eluted by a linear gradient down to 25% saturated ammonium sulfate. This provides a two-fold purification to yield a preparation in which the protein migrated in a single band by polyacrylamide gel electrophoresis. The isolation procedures used for ATase, and P_{II} are similar to those described previously by Caban and Ginsburg (Biochem. 15, 1970) and Adler et al (Adler, Purich and Stadtman, J.B.C. 250, 6264, 1975).

(10) Interaction between actin from rabbit skeletal muscle and phalloidin, a cyclic peptide isolated from the green deathcap toadstool Amanita phalloides was studied by measuring the changes observed in viscosity, optical density and actin fluorescence spectrum. The results show that : (i) Phalloidin binds to F-actin at a 1:1 stoichiometry (one phalloidin to one actin subunit). The binding is accompanied by a change in both absorbance and fluorescence caused by phalloidin induced actin conformational changes. The timecourse of this reaction is biphasic and their half-times are in the millisecond range. In the presence of 0.6 M KI, a reagent normally dissociates the F-actin to G-actin, the viscosity of phalloidin-F-actin is reduced slightly, which indicates that the phalloidin-F-actin complex cannot be dissociated to G-actin. (ii) No change is observed in both absorbance and fluorescence caused by the addition of phalloidin to G-actin. (iii) Under the polymerization conditions for G-actin, the presence of phalloidin will shorten the half-time for polymerization from 600 sec to 140 sec. The mechanisms for the acceleration of G-actin polymerization and the conversion of F-actin to a nondissociated form by phalloidin are being investigated.

(11) Inhibition mechanisms by amino acids, such as D- and L-alanine, D- and L-valine and glycine, on the biosynthetic reaction catalyzed by the Mg^{2+}-supported unadenylylated glutamine synthetase have been extensively studied. The results of both pre-steady-state and steady-state experiments indicate that the inhibition mechanism is consistent with the (oversimplified) scheme shown here:



Scheme III

where all Ks are dissociation constants and k is catalytic rate constant. The steady-state rate expression (equation 13) for scheme III can explain the non-straightforward inhibition pattern exhibited by these amino acids.

$$v_i = V_m \frac{[S](\alpha K_{i,1} + \beta[I])}{[S](\alpha K_{i,1} + [I]) + \alpha K_s K_{i,1}\left(1 + \frac{[I]}{K_{i,1}} + \frac{[I]}{K_{i,2}} + \frac{[I]^2}{\gamma K_{i,1} K_{i,2}}\right)} \quad (13)$$

Scheme III requires separate binding sites for glutamate and the amino acid inhibitors. This requirement is supported by the protein intrinsic fluorescence changes induced by amino acid binding as detected with a stopped-flow machine. The relative fluorescence changes are:

$MgE_{1.0}$ + Glu(50 mM)	(decrease)	360 mv
$MgE_{1.0}$ + L-Ala (100 mM)	(decrease)	360 mv
$MgE_{1.0}$ + D-Val (100 m)	(decrease)	320 mv
$MgE_{1.0}$Glu (20 mM) + L-Ala (100 mm)		0 mv
$MgE_{1.0}$Glu (100 mM) + L-Ala (100 mM)	(increase)	80 mv
$MgE_{1.0}$Glu (50 mM) + D-Val (100 mM)	(increase)	200 mv
$MgE_{1.0}$Glu (100 mM) + D-Val(100 mM)	(increase)	260 mv
$MgE_{1.0}$Glu (100 mM) + D-Val(100 mM)	(increase)	380 mv

These data indicate the Glu, L-Ala and D-Val bind at separate sites from from each other. The results of additional fluorescence experiments indicate that Gly can bind independently from L-Ala and D-Val. The dissociation constants for some of these amino acids are:

$MgE_{1.0}$ + L-Glu	15 mM
$MgE_{1.0}$ + L-Ala	>60 mM
$MgE_{1.0}$ + D-Val	>50 mM
$MgE_{1.0}$ATP + L-Ala	30 mM
$MgE_{1.0}$ATP + Gly	25 mM
$MgE_{1.0}$ATP + D-Val	8 mM
$MgE_{1.0}$ATP NH_3 - L-Ala	20 mM
$MgE_{1.0}$ATP NH_3 + D-Val	8 mM

These data show that ATP exhibits relatively strong synergism with all the amino acids studied here. When the experiments were carried out to determine the inhibitory effect on each step in the catalytic cycle (Scheme I), the results show that: (i) The presence of L-Ala does not significantly affect the amplitude due to intermediates 1 and 2 ($\alpha \approx 1.2$, see Scheme III). This is in agreement with the fact that a fraction of inhibition by L-Ala observed by steady-state assay is almost independent of Glu concentration. These data clearly suggest that L-Ala and Glu bind at a separate site.

(ii) L-Ala inhibits the NH_3 attacking step on intermediate 2. However, L-Ala does not completely inhibit this reaction, it merely decreases the rate constant significantly for the conversion of intermediate 2 by NH_3 to products. The fact that L-Ala-intermediate 2 can react with NH_3 to form products is consistent with an intercept of 1.15 at the ordinate when one plots [fractional inhibition]$^{-1}$ vs [L-Ala]$^{-1}$. The dissociation constant observed for L-Ala from this process is 20 mM which is in good agreement with the value of 20 mM determined for $MgE\underset{1.0}{---}ATP \cdot NH_3$ and the value of 15 mM obtained for K_i (at lower L-Ala concentration). (iii) D-Val inhibits the formation of intermediates 1 and 2 and shows strong antagonism (α = 15) between D-Val and Glu binding. However, once intermediate 2 is formed, the rate of NH_3 attacking reaction is essentially independent of the presence of D-Val except the D-Val concentration is very high (e.g. half-time is double at 100 mM D-Val). In addition, the data also indicate that there are two D-Val binding sites, with the second binding site being very weak. (iv) The inhibition pattern of Gly is similar to both L-Ala and D-Val. At high Gly concentration, both formation of intermediates 1 and 2 and the rate of NH_3 attack on intermediate 2 are inhibited. (v) The inhibition pattern exhibited by D-Ala is similar to D-Val and the inhibition pattern of L-Val is similar to L-Ala. The final analysis of all data indicate that there are two amino acid binding sites in addition to the glutamate site; one for L-amino acid and one for D-amino acid (Gly can bind to both sites). L-amino acid can bind weakly to the D-amino acid site and vise versa. The binding to the L-amino acid site will result in a decrease in rate for the reaction between intermediate 2 and NH_3, while the binding to the D-amino acid site will antagonize the binding of glutamate.

(12) Inhibition studies with AMP and γ-glutamyl hydroxamate on the biosynthetic activity exhibited by Mg^{2+}-supported unadenylylated glutamine synthetase show that there are two binding sites for each inhibitor. Lineweaver-Burk plots for the steady-state data indicate a mixed inhibition pattern for γ-glutamyl hydroxamate with respect to glutamate and for AMP with respect to ATP. Stopped-flow fluorometer studies show that AMP exhibits a strong antagonism with respect to ATP binding while γ-glutamyl hydroxamate strongly inhibits the conversion of intermediate 1 to intermediate 2.

Significance to Biomedical Research:

The object is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties and to elucidate principles of interactions between effectors, regulators and proteins.

Proposed Course of Research:

(1) To further explore the physical and chemical properties of unadenylylated and adenylylated glutamine synthetase. In particular, we will utilize the fast reaction technique, H^1 and p^{31} NMR methods to elucidate the roles of inhibitors and to identify the intermediates in the catalytic cycle.

(2) To further expand the cyclic cascade model and to elucidate the

3/

mechanism of the cascade system which regulates the activity of glutamine synthetase. The experiments will be designed particularly to test the validity of the cyclic cascade model.

(3) Kinetic and mechanistic study of glutamate synthase will be carried out. Both steady-state and presteady-state data will be used to elucidate the mechanism. In addition, the oxidation states of flavin and iron-sulfur cluster will be determined and attempts will be made to separate the two dissimilar subunits under mild conditions which preserve the iron-sulfur and flavin chromophores and allow reconstitution of active enzyme.

(4) To develop the laser heating temperature-jump machine and a slow heat exchange temperature-jump machine to cover the nanosecond and second range respectively.

Publications:

S. G. Rhee, M. I. Greifner and P. B. Chock. Determination of Adenosine 5'-Triphosphate by the Luciferin-Luciferase System with a Stopped Flow Spectrophotometer. Anal. Biochem. 66, 259-264, 1975

S. G. Rhee and P. B. Chock. Mechanistic Studies of Glutamine Synthetase from Escherichia coli: Kinetic Evidence for Two Reaction Intermediates in Biosynthetic Reaction. Proc. Nat. Acad. Sci. U.S.A. 73, 476-480, 1976

S. G. Rhee and P. B. Chock. Mechanistic Studies of Glutamine Synthetase from Escherichia coli: Kinetics of ADP and Orthophosphate Binding to the Unadenylylated Enzyme. Biochem. 15, 1976

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E. R. Stadtman, P. B. Chock and S. P. Adler. Metabolic Regulation of Coupled Covalent Modification Cascade System. in Shaltiel (Ed.): Fourth International Symposium on Metabolic Interconversion of Enzymes. New York, Springer-Verlag, 1976.

S. G. Rhee, P. B. Chock and E. R. Stadtman. Mechanistic Studies of Glutamine Synthetase from Escherichia coli: An Integrated Mechanism for Biosynthetis, Transferase, ATPase Reactions. Biochim. 58, 1976

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00203-03 LB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cellular Regulation of Enzyme Levels

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Pehlke	Clinical Associate	LB NHLI
OTHER:	Cynthia Oliver	Microbiologist	LB NHLI
	Earl R. Stadtman	Chief, Lab. of Biochem.	LB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.1	.8

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this investigation is to discover a system in the procaryote E. coli with which to study the mechanisms and regulation of protein degradation. To this end, growing E. coli were perturbed by starvation for a carbon or nitrogen source, or by conditions designed to provoke the "stringent response". As confirmed in this laboratory, these conditions have been shown to increase protein degradation as measured by the release of radiolabelled amino acids from intact proteins. The intent of this investigation is to specify which proteins are degraded and how the process is controlled. Techniques utilized have exploited polyacrylamide gel electrophoresis, isotopic studies, and the enzymatic assay of functional proteins.

PHS-6040
(12-75)

Project Description:

Control of cellular enzyme concentration is an important mechanism for the regulation of metabolism. The level of an enzymatic protein reflects not only the rate of its de novo synthesis but also the rate of its degradation. While understandings of the protein synthetic system have emphasized its importance in cellular metabolism, comparatively little is known about the process by which proteins are degraded. Since metabolic energy, RNA synthesis, and protein synthesis are required for protein degradation there is a high probability that this process, too, plays an important cellular role. The objectives of this investigation are to elucidate the basic cellular processes underlying protein degradation.

Major Findings:

I. In E. coli, the transition from growth to non-growth conditions is accompanied by an increase in the rate of protein degradation as assessed by release of radio-labelled amino acids from intact proteins. This change is noted when the cells are starved for a nitrogen or a carbon source, or when the cells are deprived of a required amino acid. Amino acid starvation induces the "stringent response" which is apparently mediated by the highly phosphorylated nucleotides, guanosine tetraphosphate and guanosine pentaphosphate.

Studies utilizing the release of ^{3}H-leucine to measure protein degradation were performed on three strains of E. coli isogeneic except at two loci. Wild type was compared with mutants carrying a temperature sensitive valyl-tRNA synthetase; one of these was stringent as was the wild type, the other was of the relaxed phenotype. By shifting the culture temperature to 40°, the temperature sensitive mutants became effectively amino acid starved, provoking the stringent response. Protein degradation was measured under nitrogen starvation and after temperature shift.

At 30°, with complete media, no difference among the strains in degradative rate was evident. With nitrogen starvation, the rate was doubled, but without strain differences. At 40°, with complete media, the wild type continued to grow and maintained a low rate of degradation. The relaxed ts mutant could not grow but did not differ from the wild type in degradative rate. The stringent ts mutant also did not grow but nearly doubled its degradative rate. Nitrogen starved cells at 40° demonstrated a marked increase in degradation in both the wild type and stringent mutant; the relaxed mutant was unable to increase degradation above its previous level.

These studies demonstrate involvement of the stringent system in at least one aspect of the protein degradation system.

II. The above cell strains, under similar provocative conditions, have been studied by a new gel technique developed for the rapid survey of protein

and enzymatic activity in crude cell extracts. Both qualitative and quantitative differences have been observed, with the appearance of new protein bands under starvation conditions. These represent either de novo synthesis or the breakdown products of previously existing proteins. Identification and quantitation of at least some bands is possible using specific enzyme assay techniques on the gel. Changes in banding patterns in response to metabolic inhibitors, inhibitors of protein synthesis, or protease inhibitors will be studied.

III. Other studies have included analysis of phosphorylated material in E. coli as a function of the stringent response in a pulse-chase type experiment. Cells were exposed to ^{32}P and the stringent response elicited by temperature shift in an appropriate mutant. Soluble material from cell extracts before and after temperature shift was electrophoresed and the gels were scanned for radioactivity. Activity was found in high molecular weight fractions before but not after the stringent response indicating either degradation, de-phosphorylation, or rapid turnover. This material was not sensitive to DNAse, RNAse, or streptomycin sulfate. Further studies have been designed to identify and characterize this phosphorylated material and to investigate the mechanism of phosphate turnover.

Proposed Course of Action:

It is well known that effective cellular enzymatic function oscillates widely with respect to changing metabolic conditions and considerable study in recent years has been devoted to ligand-induced changes in enzymatic activity as well as regulation of enzyme level by differential synthesis. However, the process by which cells preferentially degrade certain enzymes under conditions of metabolic stress for the reutilization of amino acids in de novo synthesis has not been extensively investigated. Therefore, to study this process it will be necessary initially to refine the techniques already developed for the detection of protein turnover and rapid screeing of enzyme level as a function of growth perturbation. Subsequent investigations will involve the isolation and purification of an enzyme or a class of enzymes which is subject to specific degradation as well as characterization of the inactivating system in order to elucidate the mechanisms of degradation and the regulation of this process.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00210-03 LB

PERIOD COVERED
July 1, 1976 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulation of the Cascade Control of E. coli Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Edgar G. Engleman Clinical Associate LB NHLI

OTHER: E. R. Stadtman Chief LB NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.1	.3

SUMMARY OF WORK (200 words or less - underline keywords)

1. Protein purification and characterization: The partially purified uridylyl removing/uridylyltransferase enzymes(s) have been sized at 140,000 Daltons. A nucleotide monophosphate binding substance from E. coli was enriched 50-60 fold by precipitation with streptomycin sulfate and ethanol fractionation.
A six fold purification of PII regulatory protein, already 200 fold enriched, was achieved by passage over a mercury-agarose column. PII survived exposure to 2-6 M urea.
2. Steady state analysis of the deadenylylation reaction suggests a reaction scheme in which ATP and α-ketoglutarate bind to adenylyl transferase independently, each enhancing the binding of the other. The binding sites of glutamine and α-KG on adenylyl transferase appear to be separate.
3. Inhibition of uridylyl removing activity by a variety of compounds such as coenzyme A and CMP is non-competitive with respect to substrate (PIID). The effects of these inhibitors and the enhancement of uridylyl removing activity by glutamine are very sensitive to pH.
4. Colorometric assays for uridylyl transferase and uridylyl removing activities have been adapted to an assay developed by S. Rhee for the the purpose of measuring PIID.

PHS-6040
(12-75)

Project Description:

Objective: In addition to regulation by cumulative feedback inhibition, glutamine synthetase from E. coli W is regulated by the covalent attachment of an AMP moiety to a tyrosyl residue in each of the enzyme subunits. The attachment and removal of the AMP is catalyzed by an enzyme known as adenylyltransferase (ATase). The activity of ATase is modulated, however by a second protein, PII, which exists in two forms. PIIA and PIID promote adenylylating activity and deadenylylating activity of the ATase, respectively. PIIA is converted to PIID by the covalent attachment of a UMP group to a tyrosine in each of its subunits. The interconversion of PIIA and PIID is catalyzed by the uridylyltransferase (UTase) and uridylyl-removing (UR) (enzymes(s). My specific goal is to study the regulation of this cascade. In order to accomplish this, the enzymes and proteins involved must be separated from one another; eventually, homogeneous preparations of each protein must be obtained in large quanitity.

Major Findings:

1. Further characterization of the UR and UTase Enzymes: Initial attempts to size the partially purified UR and UTase activities over agarose and sephadex columns resulted either in complete loss of activities or in multiple widely distributed small peaks of activity. However, in the presence of .5M KCl, 80% of the activities were recovered in a single peak of estimated size 140,000 Daltons, based on the elution positions of hemoglobin, hexokinase, lactic dehydrogenase, ATase and pyruvate kinase. As reported previously, streptomycin sulfate precipitation followed by DEAE cellulose chromatography, ammonium sulfate fractionation and chromatography over Sepharose AH have yielded a preparation enriched approximately 500 fold for both activities. However, affinity chromatography (passage over ATP, ADP, CDP and UDP agarose columns), organic solvent fractionation, and passage over a mercury-agarose column have all failed to increase enrichment.

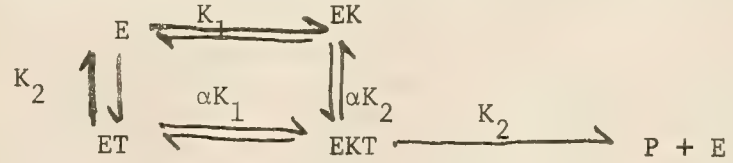
2. Further characterization of a substance which binds nucleotide monophosphates: A soluble complex, which inhibited UR activity, was isolated from crude E. coli homogenates and shown to consist of CMP and UMP bound to a larger as yet undefined compound. CMP and UMP as well as other nucleotide monophosphates were found to be potent inhibitors of the UR activity, but the nature of the beinding substance has remained uncertain. Attempts at enrichment of this substance are made difficult by the lack of a direct assay. Its presence can only be ascertained when it is bound to CMP or UMP as reflected by non-dialyzable inhibition of UR activity. Precipitation of crude homogenates with 1% streptomycin sulfate at pH 6 results in recovery of 85% of the inhibitor complex in the resuspended pellet and 20-fold enrichment. An additional 3 fold purification is obtained by precipitation with 50% ethanol followed by resuspension in buffer. Finally, the complex survives ammonium sulfate fractionation and 80-90% is precipitated in 50% ammonium sulfate. A single passage of crude extract over Sephadex G-100 yields an inhibitory peak in the exclusion fraction. However, re-passage of this peak over the same

column results in complete loss of non-dialyzable activity, even if the column is pre-equilibrated with CMP. Passage over ion exchange columns and agarose columns also result in complete loss of activity.

3. Enrichment of PII preparations over Mercury-agarose and survival in urea: Homogeneous preparations of the regulatory protein PII have been obtained in low yield only. Therefore, additional methods of PII separation were sought in order to provide higher yields of enriched preparations. Unlike the majority of proteins, PII is devoid of sulfhydryl-containing amino acids. Therefore, passage over a mercury-agarose column should result in regardation of most proteins on the column and early elution of the PII protein. This was found to be true. A partially enriched preparation of PII (following ammonium sulfate fractionation and passage over DEAE-cellulose and agarose columns) was passed over mercury-agarose at room temperature and six-fold purification was achieved with 90-100% recovery of PII activity. No further purification was achieved with pre-treatment with boryl hydride or 2 to 6 M urea, used to increase exposure of sulfhydryl groups. Of interest, however, is the observation that 90% of the PII activity was recovered following overnight exposure to 2M urea and 65% recovered after exposure to 6M urea. If dissociation of the subunits of PII takes place under these conditions, then the study of subunit-subunit interraction will be possible.

4. Regulation of the Cascade by Metabolite Effectors: We have extended our studies of the effects of metabolites on the respective enzymatic steps in the cascade control of glutamine synthetase. UR-UTase, PIIA, PIID, ATase and glutamine synthetase enzymes were isolated for these studies and the role of effectors in each step examined. We had previously observed the reciprocal effects of α-ketoglutarate and glutamine at every step in the cascade. The effects of these two ligands on PIID supported deadenylylation and on PIIA supported adenylylation have now been studied in more detail. Steady state kinetic analysis indicates a mixed inhibition pattern for glutamine with respect to α-ketoglutarate in the deadenylylation reaction as well as for α-ketoglutarate with respect to glutamine in the adenylylation reaction. These data are interpretated to indicate separate binding sites for α-KG and glutamine on adenylyl transferase.

By varying simultaneously the two required effectors (ATP and α-KG) for deadenylylation, it was observed that the concentration of ATP required for 50% activity varies inversely with the α-KG level and vice versa. Steady state analysis (by P. B. Chock) of the data suggests the following reaction scheme for deadenylylation:



It is proposed that ATP ("T") and α-KG ("K") can bind independently to the ATase enzyme ("E") and that the binding of one effector enhances the binding of the other by a factor (α). The rate expression derived from this mechanism is

$$V^{-1} = \frac{1}{K_2[Eo]} \left\{ \frac{1}{[T]} \left(\frac{\alpha K_1 K_2}{[K]} + \alpha K_2 \right) + \left(1 + \frac{\alpha K_1}{[K]}\right) \right\}$$

from which one obtains the dissociation constants for α-KG (0.1mM) and ATP (.01mM) and the enhancement factor α (20).

We previously reported that a number of compounds, such as nucleotide monophosphates and coenzyme A, were potent inhibitors of the UR activity. Further study of these effects reveals that in each case the inhibitory potency is approximately ten-fold greater at pH 7.6 than at pH 8.6. Enhancement of UR activity by glutamine was observed at concentrations as low as .5mM at pH 7.6, but at pH 8.6 up to 3 mM glutamine had no effect. The presence of glutamine did not alter the potency of these inhibitors. The concentration of CMP, for example, resulting in 50% inhibition of UR in the absence of glutamine also resulted in 50% inhibition of glutamine stimulated UR activity. The maximum inhibition obtained by addition of any one inhibitor was 80%, suggesting that the mechanism of inhibition is non-competitive with respect to substrate (PIID). Surprisingly, when CMP and UMP were added together to the UR assay, their inhibitory effects were not greater than CMP or UMP added alone, implying that their effects are antagonistic.

5. Colorometric assays for UR and UTase activities: The assays previously used to determined UR and UTase activities measured the release of radioactive AMP from glutamine synthetase. The expense incurred in the synthesis of 14C-AMP-glutamine synthetase is considerable and, therefore, a colorometric assay is desirable, provided linearity and sensitivity can be maintained. A colorometric assay developed by S. Rhee for PIID measurement has been modified and successfuly adapted for measurement of UR and UTase activities. This is accomplished by first incubating UR or UTase enzymes with their respective substrates and effectors and then transferring aliquots into a colorometric PIID assay.

Significance to Biomedical Research: The object of this work is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties and to elucidate principles of interaction between effectors, regulators and proteins.

Proposed Course of Research: (1) Further attempts to purify and characterize the UR and UTase activities. (2) Further attempts to purify and characterise the CMP/UMP binding substance. (3) Use of steady state analysis to elucidate a reaction scheme in the uridylyl transferase reaction by varying α-KG and ATP in the presence of constant UTP and PIIA (analagous to that derived for the deadenylylation reaction). (4) Studies of the effects of glutamine and α-ketoglutarate on the UTase reaction in an effort to determine whether or not glutamine and α-KG bind at the same or separate sites on the UTase protein.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00211-03 LB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulation of Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. Z. Smyrniotis — Chemist — LB NHLI
E. R. Stadtman — Chief, Laboratory of Biochemistry — LB NHLI

OTHER: Mary Wittenberger

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.2	1.3

SUMMARY OF WORK (200 words or less - underline keywords)

Heterologous interactions between adenylylated and unadenylylated subunits of glutamine synthetase do not affect the catalytic potential (Vmax) of unadenylylated subunits, but they do affect the affinity of adenylylated subunits for the substrate glutamate.

The capacity of several monovalent cations to activate or inhibit the biosynthetic activity of glutamine synthetase indicates that there is at least one relatively non specific binding site on the enzyme for monovalent cations that leads to activation of the enzyme and in addition there is an inhibitor site that is relatively specific for Cs^+, Li^+ and Rb^+.

Each subunit of glutamine synthetase possesses two kinds of binding sites for amino acids. One site is non-specific for all D-amino acids and the other is specific for L-alanine and L-serine; glycine can bind to both sites.

PHS-6040
(12-75)

Project Description:

Objectives: To determine if heterologous interactions between adenylylated and unadenylylated subunits of glutamine synthetase affect catalytic parameters, to study the mechanisms of action of monovalent cations on glutamine synthetase and to study the cumulative feedback inhibition effects of various amino acids.

Major Findings: Glutamine synthetase preparations containing an average of 1 to 11 adenylylated subunits were prepared by incubating unadenylylated glutamine synthetase for various periods of time in the presence of ATP, glutamine and adenylyltransferase. The adenylylation reaction was stopped at various stages by 200 fold detection of the reaction mixtures with cold buffer at pH 6.6. Aliquots of the diluted samples were then used for assay of biosynthetic activity at various concentrations of glutamate. The apparent Km for glutamate and specific activities were determined from double reciprocal plots of initial rate data versus the glutamate concentration. The data showed that in the presence of Mg^{2+} when only unadenylylated subunit activity is measured, the specific activity of the enzyme decreases linearly as a function of increasing state of adenylylation. Moreover, the apparent Km of adenylylated subunit for glutamate measured in the presence of Mn^{2+} is a nearly linear function of the average state of adenylylation. These results show that heterologous subunit interactions do not affect the catalytic potential of unadenylylated subunits but they do affect the apparent affinity of adenylylated subunits for glutamate.

The biosynthetic activity of unadenylylated subunits in the presence of Mg^{2+} is stimulated 40-50% by 0.3 M concentrations of the monovalent cations K^+, Na^+, and $CH_3NH_3^+$ and by low concentrations (0.05-0.1 M) of Cs^+, Li^+ and Rb^+; however high concentrations of the latter three cations are inhibitory; triethanolamine and $(CH_3)_2NH_2^+$ are without effect. The results indicate that there is at least one nonspecific relatively high affinity activator site for monovalent cations on each glutamine synthetase subunit and in addition there is a low affinity inhibitor site that is relatively specific for Cs^+, Li^+ and Rb^+. These effects are not due to ionic strength since even high concentrations of triethanolamine and $(CH_3)_2NH_2^+$ have no effect on enzyme activity.

In continuing studies on the cumulative feedback effects of multiple inhibitors it was found that all D-amino acids are inhibitors of glutamine synthetase whereas the only L-amino acids capable of inhibitng the enzyme are L-alanine, L-serine, L-tryptophon and L-histidine. These data together with the effects of amino acid binding on the intrinsic fluoresence of glutamine synthetase (see annual report of P. B. Chock, et. al.,) indicate that there are two kinds of amino acid binding sites on the enzyme, one is specific for D-amino acids and the other specific for L-alanine or L-serine. Glycine can bind to both sites.

Proposed Course of Action:

Studies on the cumulative feedback inhibitor of multiple metabolites will be continued with the ultimate aim of establishing how many separate binding sites there are on the enzyme for the different effectors. The fluoresence technique will also be used to study the effects of monovalent cations in great detail.

Significance to Biomedical Research:

Defects in mechanisms of cellular regulation of enzyme activity are the basis of certain metabolic diseases. Cumulative feedback inhibition is one important mechanisn by which key enzymes are regulated. Because of its central role in nitrogen metabolism glutamine synthetase serves as a unique model to elucidate basic principles that underlie the cumulative feedback control mechanism.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00212-05 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemical Genetics of NH_3-Assimilatory Enzymes in E. coli K_{12}

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mary Anne Berberich Chemist LB NHLI

COOPERATING UNITS (if any)

Wayne B. Anderson, Ph. D. Lab. Molecular Biology, NCI/NIH
Saul Brusilow, M.D. Prof. Pediatrics, The Johns Hopkins School of Medicine

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	.3

SUMMARY OF WORK (200 words or less - underline keywords)

(1) Selection of, and genetic studies on, mutants of glutamine synthetase, glutamate synthase and glutamate dehydrogenase in E. coli K_{12}.

(2) Studies on the mechanism of resistance to drugs such as methionine sulfoximine.

(3) Studies on the manifestation of regulatory interrelationships among NH_3-assimilatory enzymes.

(4) Work toward the development of a reliable microbiological assay for plasma glutamine.

PHS-6040
(12-75)

Project Description;

Objectives: (1) To isolate and characterize biochemically, mutants in which the activity of glutamine synthetase, glutamate synthase or glutamate dehydrogenase is affected and to conduct genetic experiments for the purpose of locating these mutations on the E. coli K_{12} chromosomal map. (2) To elucidate the mechanism of methionine sulfoximine resistance and its effect on the derepression of glutamine synthetase. (3) To develop methods to study possible regulatory inter-relationships among the NH_3-assimilatory enzymes in E. coli K_{12}. (4) To develop a reliable microbiological assay for plasma glutamine.

Major Findings:

1. Studies with Klebsiella aerogenes suggest that constitutivity of glutamine synthetase can result from mutations affecting enzymes involved in the chain of biochemical events leading to modification of this enzyme by adenylylation (Foor et al., PNAS, 72:4844, 1975). It is interesting that the genetic loci, gln B, and gln D, apparently designating the P_{II} regulatory protein and uridyltransferase respectively, have been tentatively assigned map positions which are approximately that determined for glutamate synthase (Berberich, Biochem. Biophys. Res. Comm., 47:1498, 1972). Another locus, gln E, presumably the structural gene for adenyltransferase has also tentatively been assigned to a map position in the region of that for glutamate dehydrogenase (Janssen et al., Abstr of Ann. Meeting of ASM, New York, 1975). This genetic juxtaposition may be significant in the scheme of regulation of NH_3 assimilation and efforts to obtain such mutations in E. coli K_{12} are being made. Of additional interest, is the fact that, in the case of Klebsiella, the gln C^- regulatory-type mutations which are now assigned positions in the gln A structural gene locus were derived from a glutamate synthase (GAT) minus parent. The constitutive phenotype does not appear to be demonstrable in a GAT+ background when episomes carrying gln C^- are introduced into K. aerogenes, presumably due to subunit mixing. Crosses between E. coli, K_{12} gln A's show a notable absence of abortive transductants which indicates that subunit mixing is negligible.

2. Attempts to correlate the phenomenon of MSO resistance with the mechanism of glutamine synthetase derepression are in progress and early work suggests that, if the structural gene for glutamine synthetase is autogenously regulated, it functions via a cooperation with some other element - perhaps a component of GAT. The observation that methionine potentiates MSO resistance is being investigated in view of the fact that methionine is a potent inhibitor of GAT. Methionine also appears to exert an effect on glutamine synthetase derepression in a GDH$^-$ GAT$^-$ double mutant. (See below).

3. Some recent findings with a temperature-sensitive t-rna synthetase mutant indicate that, at the restrictive temperature, the activity of glutamate synthase is elevated as is that of glutamine synthetase (LaPointe et al., J. Bact. 123:843, 1975). The interpretation offered is that charged

glutamyl-t-rna is a co-repressor in the regulation of both of these enzymes. However, there are other observations which appear to complicate this hypothesis of unity in control of these enzymes. Glutamine synthetase is maximally derepressed in cells grown with 60 mM glutamate as N source, conditions where glutamate synthase can be shown to be completely repressed. Also, cells grown in the presence of cyclic-AMP are derepressed for glutamine synthetase and repressed for glutamate synthase.

Altered patterns of glutamate derepression of glutamine synthetase, which appear independent of the state of adenylylation, are being studied in revertants of gln A$^-$ mutants. The differences observed between limiting-NH_3 derepression and glutamate derepression, marked in some of these strains, may be a manefestation of the involvement of an additional component in the derepression of glutamine synthetase. A comparison of glutamine synthetase levels between the wild-type organism and a GAT$^-$ GDH$^-$ double mutant show differences which depend on culture conditions. In the double mutant, glutamine synthetase is not as completely repressed as that in the wild-type strain when cells are grown in the presence of 50 mM NH_4Cl with 10 mM glutamine. Moreover, when L-methionine is added to this medium at a final concentration of 200 µg/ml, derepression of glutamine synthetase occurs in the mutant. Under these conditions, where derepression of glutamine synthetase appears to be maximal for the double mutant, enzyme from wild-type cells is completely repressed. While derepression of the wild type enzyme in high glutamate depends on the absence of NH_4Cl, that for the double mutant does not. However, maximal glutamate derepression of glutamine synthetase in the mutant is approximately 25% that of wild type.

Repression of glutamate dehydrogenase when cells of *E. coli* K_{12} W_{3102} are grown on high glutamate requires the presence of NH_4Cl in the medium. In the absence of NH_4Cl, amino acid induction of glutamate dehydrogenase occurs in the presence of 60mM glutamate. These are the conditions where derepression of glutamine synthetase in wild type cells is maximal. The relationship of these enzymes is being investigated further.

In order to assess whether subunit sharing among the NH_3 assimilatory enzymes might be involved in an inter-regulatory scheme, some immunologic studies were undertaken. Antibody was prepared in the rabbit against purified glutamate synthase which, on the basis of the observations discussed above, appeared to be the most likely candidate for this possibility. The γ globulin fraction containing anti-glutamate synthase was titrated vs its eliciting antigen and was found to be neutralizing and precipitating and the antigen-antibody complex fixed complement in the micro CF test. Attempts to demonstrate a cross-reactivity with purified glutamine synthetase (n 1 - 12) were negative by all these criteria. However, when anti-glutamate synthase γ globulin was added to crude extracts before measuring glutamine synthetase activity in the transferase assay, some effects such as co-precipitation and re-activation of gln A$^-$ enzyme could be observed. These findings are being investigated further, since some association between the two proteins might exist in vivo.

The hypothesis that glutamate synthase might exert an allosteric effect on glutamine synthetase, as reported for other systems (Uyeda and Luby, J. Biol. Chem., 249:4562, 1974) will be examined more directly in vitro. It should be possible to observe energy transfer between appropriate fluorescing derivatives of the two enzymes if this is the case. Also, the effect of glutamate synthase on the kinetics of the glutamine synthetase reactions will be examined.

The experiments of Tyler et al., (PNAS, 71:225, 1974) which demonstrated the positive effect of glutamine synthetase upon transcription of the hut operon in vitro, and the apparently reciprocal effects of C-AMP on glutamine synthetase and glutamate synthase (Prusiner et al., PNAS, 69:2922, 1972) suggested that the relationship between cyclic-AMP, cyclic-AMP binding protein (CRP), glutamate synthase and glutamine synthetase should be examined further. Crude serum prepared against a purified preparation of CRP (Anderson et al., J. Biol. Chem., 246:5929, 1971) was obtained from Wayne Anderson and the γglobulin fraction tested in various ways against the two NH_3 assimilatory enzymes. A precipitin band could be demonstrated between anti-CRP γglobulin and purified glutamine synthetase (n 1 and 11). This band is cross-reactive with the anti-CRP vs CRP precipitin band and is homologous with a minor band which can be seen on Ouchterlony double-diffusion slides between anti-glutamine synthetase and glutamine synthetase. Anti-glutamine synthetase antibody does not show a precipitin band vs. CRP. Anti-CRP γ globulin has been adsorbed with purified glutamine synthetase. Both the cross-reactive band vs glutamine synthetase and the precipitin band vs CRP are removed by this treatment. Anti-CRP γ globulin has no effect on glutamine synthetase activity and very little effect (15% inhibition with maximal amounts of antibody) on the cyclic-AMP binding activity of CRP. For this reason, in order to assess the biological significance of this immunologic cross-reactivity, arrangements have been made to collaborate in a study of the effect of adsorbed anti-CRP γ globulin upon the cyclic-AMP stimulated in vitro transcription of the gal operon which is inhibited 80% by anti-CRP antibody.

The anti-CRP γ globulin does not react with glutamate synthase (nor does anti-GAT γ globulin react with CRP) or with purified phosphoribosyltransferase the first enzyme of the histidine pathway which has also been implicated in autogenous regulation.

4. I have been engaged in a collaborative study with members of the Pediatrics Department, The Johns Hopkins School of Medicine. We are trying to develop a microbiological assay for plasma glutamine which would aid in screening for a type of hyperammonemia prior to the onset of symptoms. So far, the mutants which I have employed do not respond to the glutamine offerings in the concentration range defined by the disease. Also, the specific glutamine analogue (MSO) which I've employed complicates the picture by giving rise to resistant forms quite readily. I would like to titrate the effective concentration of inhibitor which will just allow growth of the bacterial lawn when the desired level of glutamine is added by means of sterile pads. Plans for the future include (a) the use of the glutamine antagonist, diazo-ozynorleucine (DON) as a possible inhibitor for

the titration method; (b) demonstration of the growth response of glutamine mutants to glutamine discs supplied to liquid culture where, for reasons as yet undetermined, these strains will exhibit growth at low concentrations of glutamine.

Proposed Course of Project: Genetic studies to refine the chromosomal regions containing the structural genes for glutamine synthetase, glutamate dehydrogenase, and glutamate synthase will continue with an emphasis on isolation of regulatory mutants. Enzymic analysis of revertants of structural gene mutants will continue. Physiological and biochemical studies on the interrelationships of NH_3 assimilatory enzymes in mutant strains will continue. The biological significance of the glutamine synthetase CRP relationship will be pursued.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00214-02 LB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Enzyme Degradation in Klebsiella aerogenes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard Fulks Staff Fellow LB NHLI

Other: E. R. Stadtman Chief, Laboratory of Biochemistry LB NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.1	.3

SUMMARY OF WORK (200 words or less - underline keywords)

When Klebsiella aerogenes were grown and then transfereed to medium containing sufficient chloramphenicol to block protein synthesis, degradation of glutamine synthetase occurred. Inactivation of the enzyme was stimulated by glucose or glycerol, and inhibited by dinitrophenol. Inactivation occurred more rapidly at higher pH values, over a range from pH 6 to 8. Inactivation also occurred more rapidly in cells originally harvested during log phase of growth than in cells incubated for several hours in stationary phase prior to harvest for resuspension and incubation in the presence of chloramphenicol.

Cell-free extracts have been prepared which inactivate glutamine synthetase at a rate similar to that observed in intact cells incubated in the presence of chloramphenicol. As observed in intact cells, enzyme inactivation in the extracts occurred more rapidly at higher pH values, and in extracts prepared from cells originally harvested during log phase of growth. Enzyme inactivation in the extracts was stimulated by some metal ions and inhibited by others. EDTA inhibited inactivation.

Klebsiella glutamine synthetase has been purified to homogeneity or near homogeneity. The purified enzyme incubated alone was stable under conditions in which it was inactivated when added to a cell extract. A boiled extract was unable to inactivate the purified enzyme.

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PHS-6040
(12-75)

Project Description:

The original objective of this project was to study the mechanism and regulation of intracellular proteolysis through study of degradation of specific intracellular enzymes.

As mentioned in last year's annual report, initial studies were concerned with the inactivation of lysine-sensitive aspartokinase that occurred in Klebsiella aerogenes in response to nitrogen limitation. Later studies focused on the inactivation of glutamine synthetase that occurred in cells which had been grown and then transferred to medium containing chloramphenicol. This report describes additional studies concerned with the inactivation and degradation of glutamine synthetase in intact cells incubated in the presence of chloramphenicol, and with a similar inactivation process in cell-free extracts.

Major Findings:

As mentioned in last year's report, when Klebsiella were grown and harvested and then resuspended and incubated in medium containing sufficient chloramphenicol to block protein synthesis, over 90% of the glutamine synthetase activity present at the beginning of incubation was lost within 3-4 hours. Glutamine synthetase antigen also declined, indicating a reduction in enzyme amount as well as enzyme activity.

Glucose or glycerol stimulated the inactivation process, and dinitrophenol inhibited inactivation. These findings suggested that degradation of the enzyme required energy.

Ammonium (chloride) also stimulated inactivation of glutamine synthetase in cells incubated in the presence of chloramphenicol. Ammonium is known to stimulate adenylylation of glutamine synthetase, but adenylylation could not, by itself, account for the current finding, because control experiments showed that the glutamine synthetase assay used was unaffected by changes in states of adenylylation of the enzyme. It is possible that ammonium stimulated adenylylation, and that adenylylation in turn rendered the enzyme more susceptible to proteolysis, but this possibility has not yet been tested. Although the mechanism by which ammonium stimulates inactivation of glutamine synthetase is not clear, additional experiments suggested that this effect requires a carbon or energy source, because ammonium had no effect when glucose was omitted from the incubation medium.

The pH of the medium also affected inactivation of glutamine synthetase. Increasing the pH from 7 to 8 stimulated inactivation, whereas lowering the pH to 6 protected the enzyme from inactivation.

Control experiments done in the absence of chloramphenicol showed that the cells could incorporate ^{14}C-leucine into glutamine synthetase antigen, or into TCA-precipitable protein, even when the label was added when no increase in glutamine synthetase activity or total cell protein was occurring.

Thus, the cells appeared to degrade glutamine synthetase, and other proteins, in the absence of chloramphenicol as well as in its presence.

In E. coli, chloramphenicol did not cause reductions in glutamine synthetase comparable to the reductions observed in Klebsiella. That the glutamine synthetases of these two organisms appear to be structurally similar suggests that the apparent difference in rates of degradation of the enzyme is due to a difference in the mechanism or regulation of proteolysis in the two organisms. This interpretation received support from the observation that in E. coli chloramphenicol also failed to cause the reduction in total cell protein that occurred in Klebsiella.. One possible, but untested, explanation for these findings is that chloramphenicol may have inhibited proteolysis in E. coli (as has been reported, PNAS, 68: 362, 1971) but not in Klebsiella.

In a typical experiment, Klebsiella previously grown, harvested, and frozen were suspended in a volume of buffer (pH 8) equal to twice the weight of the cells. After sonication and centrifugation, the resulting cells extract was incubated with shaking at 37°. At various time, aliquots of the extract were removed and assayed for glutamine synthetase anzymatic activity, and occasionally for glutamine synthetase antigen.

Early experiments showed that failure to centrifuge the extract prior to incubation did not increase the rate of inactivation of endogenous glutamine synthetase. Inactivation occurred at 37° but not 0°. Failure to mix the extract during incubation reduced the rate of inactivation.

As has been found to intact cells incubated in the presence of chloramphenicol, the rate of decline of glutamine synthetase activity (and antigen) increased as the pH was raised over a range from 6 to 8.

A second similarity between the inactivation of glutamine synthetase in extracts and in intact cells in the presence of chloramphenicol was that inactivation occurred more rapidly when the cells were harvested during log or onset of stationary phase than when the cells were harvested after several hours of incubation in stationary phase.

Because studies in intact cells had suggested that degradation of the enzyme required energy, the effect of adding ATP to the extract was assessed. High levels of ATP (10-20 mM) stimulated both the loss of glutamine synthetase activity and antigen. However, control experiments showed that these levels of ATP could inactivate purified glutamine synthetase. Additional experiments are needed to determine whether ATP can physiologically stimulate inactivation and degradation of the enzyme.

A number of metal ions affected inactivation of glutamine synthetase in the cell extracts. Manganese, nickel, or cobalt (1.5 mM) protected glutamine synthetase from inactivation. Manganese caused partial protection when added at 10^{-4} or 10^{-5} M. Magnesium or calcium (1.5 mM) has little effect on inactivation. Zinc, iron, or copper stimulated inactivation of the enzyme.

EDTA protected the enzyme from inactivation. When nickel or cobalt, but not manganese, magnesium, calcium, zinc, iron, or copper was added to the extract at the same concentration as the EDTA, the protective effect of EDTA was overcome, and inactivation of the enzyme occurred.

For control experiments concerning the stability of purified glutamine synthetase under the conditions of enzyme inactivation in extracts, and for other experiments below, glutamine synthetase was purified from Klebsiella. The procedure used was adapted from the one currently being used in this laboratory for the purification of glutamine synthetase from E. coli, and from the one used by Deleo and Magasanik for Klebsiella (J. Bact. 121: 313, 1975). Acrylamide disc gel electrophoresis of the enzyme finally obtained gave one major band, and one faint band, when stained for protein. Both bands contained glutamine synthetase enzymatic activity.

When this purified Klebsiella glutamine synthetase was incubated for 4-1/2 hours at 37° at pH 8 in the presence of 1-2 mM Mg, less than 20% of the enzyme activity was lost. Under these same conditions, over 80% of the enzyme activity was lost in an extract containing endougenous enzyme. The extract could also inactivate the purified glutamine synthetase, but not when the extract was boiled prior to the addition of the purified enzyme.

These studies suggest that the cell extracts prepared from Klebsiella contain a previously unrecognized enzyme capable of inactivating, and possibly degrading, glutamine synthetase.

Proposed Course of Research:

1. Perform additional experiments to determine whether ATP might act physiologically to stimulate inactivation and degradation of glutamine synthetase in cell extracts.

2. Determine whether protease inhibitors affect the inactivation of glutamine synthetase in cell extracts.

3. Attempt to purify the components(s) of the cell extract that inactivates glutamine synthetase.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00215-01 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Phosphofructokinase in Tetrahymena pyriformis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paulette W. Royt	NIH Postdoctorate Fellow	LB	NHLI
OTHER:	Earl R. Stadtman	Chief, Lab. of Biochem.	LB	NHLI

COOPERATING UNITS (if any)

NIAID, NIH

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.1	.3

SUMMARY OF WORK (200 words or less - underline keywords)

Phosphofructokinase has been studied in Tetrahymena pyriformis. The level of this enzyme in glucose-grown organisms is greater than that in cells grown without glucose. Starvation of glucose-grown cells leads to a very rapid loss of PFK activity, indicating an enzyme-mediated reaction. In crude cellfree extracts, enzyme activity is stabilized by ATP. Mg^{++} and Ca^{++} decrease phosphofructokinase activity, whereas, Na^{+} increases activity. Attempts to purify phosphofructokinase from this organism resulted in enzyme activity associated with large yet unindentified particles.

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PHS-6040
(12-75)

Project Description:

Objectives: The activity of an enzyme in a cell is dependent on the number of enzyme molecules present and on the regulation of those molecules. The rate of synthesis of the enzyme and the rate of its degradation determine the level of the enzyme molecules in the cell. The enzyme activity may be controlled by the intracellular concentration of substrate and allosteric modifiers, and by alterations in the enzyme molecule itself. Covalent modification of the protein may result from the action of another enzyme. The protein-protein interaction of enzyme subunits represents noncovalent modification.

Phosphofructokinase (PFK), a major glycolytic enzyme, has been studied in numerous organisms. It is well documented that this enzyme is allosterically activated and inhibited by various metabolites. The stabilizing effect of PFK activity by substrates and products indicates that intramolecular interactions contribute to PFK activity. It has more recently been determined that PFK in rat liver and mouse skeletal muscle is subject to covalent modification via kinase activity. The phosphorylated, active form of the enzyme is dephosphorylated via phosphatase activity. Preliminary experiments in this laboratory have suggested that PFK from the protozoan, Tetrahymena pyriformis, is regulated via enzyme activity. It has been the purpose of this project to further investigate regulation of PFK in Tetrahymena pyriformis.

Major Findings:

I. Levels of activity of various enzymes: Activities of the following enzymes were assayed in crude cell free extracts of Tetrahymena, harvested during logarithmic phase of growth and stationary phase, grown in the presence and in the absence of glucose: pyruvate kinase, lactic dehydrogenase, phosphoglucose mutase, malic dehydrogenase, hexokinase, and PFK. The most significant difference in the activity of an enzyme in the crude extracts occurred with PFK. Initially it was observed that logarithmic phase, glucose-grown cells have greater levels of this enzyme than do cells grown in the absence of glucose.

II. The effect of starvation on PFK activity: Glucose-grown cells were transferred from rich growth medium to sterile buffer. Measurement of PFK activity in the starved cells revealed that the enzyme activity dropped precipitously during the first minutes of starvation. This rapid decrease in activity reflects an enzyme mediated reaction.

III. The in vitro stability of PFK: It was found that PFK activity in crude cell free extracts was stabilized by the addition of 1 mM ATP. Neither 20 mM NaF nor 0.01 mM cyclic 3',5'-AMP had an effect on initial enzyme activity or on the activity after 4 hr incubation at 4C. KCl at 0.1 M and 0.2 M significantly decreased PFK activity, whereas NaCl at the same concentrations increased activity. $MgCl_2$ at concentrations of 2 to 10 mM caused a progressive decrease in the initial activity of PFK. The subsequent rate of loss

of activity did not change. Extracts prepared in 0.5 mM $MnCl_2$ and in 0.25 mM $CaCl_2$ had reduced levels of PFK activity.

Knowing that Mg^{++} and Ca^{++} are activators of pyruvate dehydrogenase phosphatase, the above findings are consistent with a phosphorylating-dephosphorylating system of PFK existing in Tetrahymena. The absence of a stimulating effect by cyclic 3',5'-AMP suggests that in Tetrahymena, as in rat liver, phosphorylation of PFK is not dependent on this metabolite.

IV. Purification of PFK: Attempts to purify PFK by affinity chromatography using blue dextran bound to Sepharose 4B failed.

Gel filtration (BioGel A150M) of ammonium sulfate precipitates of crude cell free extracts revealed PFK activity to be associated with a very large particle of approximate molecule weight, 150,000,000. Gel filtration of crude cell free extracts resulted also in a large particle with PFK activity as well as with pyruvate kinase and aldolase activity. Less consistently, pyruvate kinase activity is found associated with the large particle following ammonium sulfate precipitation.

The nature of the large particle is presently not known. PFK may be aggregated to form a very large complex. It is also possible that PFK is closely associated with other glycolytic enzymes forming a multihead enzyme complex. Also, PFK molecules, isolated or bound to other enzyme molecules, may be attached to nucleic acid or membrane particles.

Proposed Course of Action:

The identification of the large particle containing PFK activity will be pursued. Bio Gel fractions will be treated with detergents and streptomycin to determine if the enzyme is bound to lipid or nucleic acid, respectively. Further gel filtration will indicate changes in size of the particle following such treatment. During the identification procedures, the activities of other glycolytic enzymes will be assayed to determine if PFK is closely associated with or bound to other glycolytic enzymes. If a PFK-containing complex is isolated, its enzymic components will be identified as well as its physical characteristics. If, on the other hand, PFK is isolated independent of other activities, it will be purified, and its physical and kinetic properties determined. Attempts will be made to fractionate Tetrahymena cells and isolate PFK-activating and PFK-inactivating fractions. Phosphorylation and dephosphorylation of PFK will then be studied using [γ-^{32}P] ATP and perhaps antibodies to PFK.

Relevance to Biomedical Research:

The use of microorganisms to study biochemical control mechanisms is well justified, for these organisms possess metabolic processes similar to those of higher organisms. Tetrahymena, a simple eukaryotic animal cell, is one microorganism which can easily be cultivated and studied in the laboratory. Knowledge of metabolic processes of this organism can be applied to

higher animal systems.

PFK, a major enzyme of glucose catabolism, is found in all forms of life. Since this enzyme constitutes an important control point of glycolysis in cells, regulation of its activity as well as of its synthesis and degradation must be stringently controlled. Such studies of the regulation of PFK can be made in various organisms. For instance, it has been found that fasted rats and diabetic rats have reduced levels of liver PFK. This reduced level in starved rats was due to an increased rate of degradation of the enzyme with little change in the rate of synthesis. Refeeding of the starved rats returned PFK levels to that of control animals by reduced rates of degradation. It is apparent that these findings can be applied to glucose utilization under these physiological conditions in humans.

It has also been suggested that PFK is involved in the high rate of anaerobic glycolysis in cancer tissue. It has been postulated that the reduced number of mitochondria in tumor cells produce less ATP to feedback and inhibit PFK. Alterations in isozymes of PFK also may contribute to the high rate of glycolysis in the tumor cells.

Knowledge of PFK regulation is of prime importance in understanding the overall control of glucose metabolism. Only more extensive study of PFK will elucidate further the role this enzyme plays in glycolysis.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00217-01 LB

PERIOD COVERED
September 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Control of in vitro Assembly of Microtubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donita L. Garland	NIH Postdoctorate Fellow	LB	NHLI
OTHER:	Earl R. Stadtman	Chief, Lab. of Biochem.	LB	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

SUMMARY OF WORK (200 words or less - underline keywords)

The assembly of microtubules is crude supernatants has been studied using inhibitors. It was found sodium fluoride prevented the assembly of microtubules in crude supernatant prepared from brain, but not the assembly of purified brain tubulin.

The effect of the particulate subfractions on assembly of microtubules was determined. The microsomal fraction contained an activity that inhibited assembly of microtubules in crude supernatant, but not the assembly of purified tubulin.

Both inhibitions can be overcome with high levels of GTP. Work is in progress to determine the mechanisms of the inhibitions and the relevance to cell control of assembly-disassembly of microtubules.

The kinetics of the reaction between colchicine and purified brain tubulin were investigated using a fluorometric assay for the formation of colchicine tubulin complex. The results support a multi-step binding reaction. Rate constants for the reaction were determined.

PHS-6040
(12-75)

Project Description:

Objectives:

I. The formation of microtubules in vitro is now possible using either supernatant prepared from brain homogenates or purified tubulin. While it appears tau protein is the only additional protein required for assembly of purified tubulin, it seems likely that other proteins are important for control of assembly-disassembly of microtubules in the cell.

There is considerable evidence indicating there is a phosphorylation-dephosphorylation cycle for tubulin, however, the role it has is not known. For this reason the effect of sodium fluoride, a known modifier of phosphate transfer enzymes, on in vitro polymerization of tubulin was studied.

Observations were made in the course of other experiments that suggested that the particulate fractions of brain may contribute to the rapid loss of the ability of tubulin to form microtubules found in non purified systems. Elucidation of this inactivation of tubulin was pursued.

II. Colchicine prevents the assembly and causes the disassembly of microtubules by its unique binding to tubulin. Kinetic and thermodynamic data obtained previously using radiolabeling techniques were inconsistent with a simple bimolecular reaction scheme. The reaction was further studied using a fluorescent technique.

Major Findings:

(1) The in vitro assembly of microtubules in crude supernatants was inhibited by sodium fluoride. The inhibition was prevented and partially reversed by high concentrations of GTP. Sodium fluoride did not inhibit the assembly of purified tubulin unless crude supernatant was added.

Utilizing radiolabeled GTP and ATP it was shown that when sodium fluoride was added to crude supernatants, there was an increase in the GMP concentration and a decreased conversion of adenine nucleotides to GTP. The effect of GMP and GDP on the in vitro assembly of microtubules was determined using viscometry to measure the extent of assembly. GMP inhibited the assembly of microtubules in crude supernatants but had no effect on the assembly of purified tubulin. GDP conversely is a potent inhibitor of the assembly of purified tubulin but supports assembly in crude supernatant. The observation that GMP does not inhibit the assembly of purified tubulin suggests either that GMP is not directly involved with control of assembly of microtubules or that an important protein has been lost during purification.

The microsomal fraction of brain contained an activity that inhibited the assembly of microtubules in crude supernatants, but not the assembly of microtubules using purified tubulin. This heat labile activity required sucrose. High concentrations of GTP prevents the inhibition.

(2) The kinetics of the association of colchicine and purified porcine brain tubulin were studied, using a fluorometric assay for the formation of the colchicine-tubulin complex. The data is consistent with the following multi-step binding reaction:

$$P + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PC$$

However, the possibility of a third step cannot be ruled out. Rate constants for some of the steps have been determined.

(3) The colchicine binding protein found primarily in brain synaptosome fractions was solubilized from crude particulate preparations. The protein which retained the colchicine binding activity after solubilization has been purified to apparent homogeneity according to disc gel electrophoresis.

Relevance to Medicine:

An understanding of the cellular control of the assembly-disassembly of microtubules would have widespread importance. Microtubules are important for many cellular functions, i.e. cell division secretion, intracellular particle movements, axonal transport and cell motility.

Colchicine, a drug which causes the disassembly of microtubules is the classic treatment for gaut. Likewise, certain malignancies have been treated with vinblastine, another compound which disrupts microtubules. Elucidation of the mechanism of cellular control may suggest alternate treatments for these diseases.

In Alzheimers disease abnormal microtubules have been observed, although the reason for these changes is not known. Studies have also suggested the possibility of microtubule dysfunction in certain diabetes.

Proposed Course of Action:

Efforts will be continued to determine the mechanism of inhibition of assembly of microtubules in crude supernatant by the microsomal fraction and sodium fluoride. It will be necessary (1) to identify the enzymes modified by sodium fluoride. (2) to determine the mechanism of GMP induced inhibition in crude supernatant and to identify and isolate any proteins involved. (3) to determine if the inhibition by the microsomal fraction is by a modification of the tubulin, cofactors or the nucleotide concentrations.

Ultimately, it is important to determine the relevance of any of these reactions to the cellular control of assembly-disassembly.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00219-01 LB

PERIOD COVERED
October 5, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Intracellular Regulation of Enzyme Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Maria J. Mazon	Visiting Scientist	LB NHLI
OTHER:	E. R. Stadtman	Chief, Lab. of Biochem.	LB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.85	0.85	

SUMMARY OF WORK (200 words or less - underline keywords)

In order to detect any changes of enzymatic activities which could indicate a regulatory mechanism for cell metabolism, several enzymes have been assayed under growth and starvation conditions.

In the first part of this work, proline oxidase, aspartate kinase and isocitrate lyase activities were measured in Saccharomyces cerevisiae grown on minimal medium and acetate but no significant changes in their specific activities could be found during stationary phase or during nitrogen or carbon or vitamin starvation.

At present, the specific activities of glucose-6-phosphate dehydrogenase, pyruvate kinase, glutamate-oxalacetate transminase, glutamate dehydrogenase TPNH-dependent and glutamate dehydrogenase DPNH-dependent are being measured on yeasts grown on minimal medium and glucose. These enzymes have been first studied during logarithmic and stationary phases of growth and are being measured now under starvation conditions.

PHS-6040
(12-75)

Project Description

Objectives: To understand the cellular mechanisms of the regulation of enzyme activity it could be a useful tool to study the way by which the cells respond to a rapid change in their environmental conditions.

It is well known that microorganisms adapt to different growth conditions by repressing the synthesis of unneeded enzymes and inducing the production of new ones. However, it is not clear what the cell response is to a nutritional shift-down, nor how the cells survive under conditions of a limited nitrogen or carbon supply. Therefore, the objective of this project is the study of the mechanisms by which cells eliminate some enzyme activities. Proposed mechanisms are: proteolytic degradation, covalent modifications, association-disassociation phenomena etc. In fact, yeasts cells produce a number of proteolytic activies and very little is known about their physiological function, their specificity, if any, and their "in vivo" action mechanisms.

Major Findings: The specific activities of the following enzymes were first studied: proline oxidase, isocitrate lyase, and aspartate kinase. These enzyme activities seem to remain constant either during stationary phase of growth or during prolonged carbon and nitrogen starvation.

However, different behaviors are being found now among the new enzymes chosen for study: glucose-6-phosphate dehydrogenase, pyruvate kinase, glutamate dehydrogenase TPNH-dependent and glutamate-oxalacetate transaminase.

The first one, glucose-6-phosphate dehydrogenase, constantly increases its specific activity during the stationary phase of growth. This increase serves both as a control of viability of cells in late stationary phase and as an indication of some specificity in the regulation of the enzyme levels since this enzyme is increased while some others remain constant as pyruvate kinase does or are decreased. Among those enzymes which are lost are the glutamate dehydrogenase TPNH-dependent and the transminase. We do not know yet the way by which those enzyme activities decrease.

In order to obtain more information about these changes, the same enzymes are being studied under different conditions to see if it is a general or specific behavior. Since both the glutamate dehydrogenase and the glutamate-oxalacetate transminase are key enzymes of nitrogen metabolism in yeast, cells with normal levels of those two enzymes have been transferred to a medium lacking nitrogen. As a control glucose-6-phosphate dehydrogenase and glutamate dehydrogenase DPNH-dependent are also being followed.

In these preliminary studies, a striking decrease in total extracted cell protein as well as a large increase in the amount of extractable lipids, have been found after a few hours of nitrogen starvation.

Proposed Course of Action: The study of those enzymes mentioned above, glutamate dehydrogenase and glutamate-oxalacetate transminase, will be continued under carbon starvation conditions. If the results are confirmed, we will try to investigate the mechanism of these decreases "in vitro" and to isolate a "modifying fraction" from crude extracts, with which to study the mechanism of the process.

Relevance to Biomedical Research: The understanding of a cellular process like the regulation of enzyme activities in microorganisms is very likely a necessary step in the knowledge of these processes in higher animals.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00220-01 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulation of Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Park	Clinical Associate	LB NHLI
OTHER:	Sue Goo Rhee	Staff Fellow	LB NHLI
	Earl R. Stadtman	Chief, Lab. of Biochem.	LB NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.6	.3

SUMMARY OF WORK (200 words or less - underline keywords)

We have used a batch type microcalorimeter to show that the substrate L-Glutamate and the feedback inhibitor L-Alanine bind to separate sites on the enzyme glutamine synthetase on the basis of additive heats of binding.

Colorimetric assays have been developed for glutamine synthetase adenylyltransferase, ATase, and the regulation protein, P_{II}. These assays are based upon ATase and P_{II}-stimulated adenylylation or deadenylylation of glutamine synthetase.

PHS-6040
(12-75)

Project Description:

Objectives: (1) Using unadenylylated glutamine synthetase in the presence of Mg^{++}, to study the binding of the substrate glutamate and the effector alanine using a calorimetric approach. (2) To develop assay methods and purification procedures for glutamine synthetase adenylyltransferase and the regulatory protein, P_{II}.

Major Findings:

(1) Glutamate and Alanine Binding to Glutamine Synthetase. Previous kinetic analysis has been inconclusive in determining whether the feedback inhibitor, L-Alanine, binds to the same site or a different site on glutamine synthetase than the substrate L-glutamate. We have used a batch type microcalorimeter to study the heats of effector binding to glutamine synthetase and have shown that the sites for glutamate and alanine are separate. Heat is produced by alanine binding ($\Delta H' \simeq 8$ kcal/mole, $K'_D \simeq 40$ mM) to the enzyme already saturated with glutamate; furthermore, the heat of binding both glutamate and alanine simultaneously to the enzyme exceeds the heat of binding either ligand alone.

(2) Assay and Purification of Adenylyltransferase and P_{II} (with S.G. Rhee)

$$GS + ATP \xrightarrow[Me^{++}]{ATase\ P_{II}} AMP\text{-}GS + PP_i \qquad (1)$$

$$AMP\text{-}GS + P_i \xrightarrow[Me^{++}]{ATase.\quad P_{II}\text{-}UMP} GS + ADP \qquad (2)$$

We have developed colorimetric assays for the glutamine synthetase adenylyltransferase, ATase (reaction 1,2) and the regulatory protein, P_{II}, in both its unmodified form (reaction 1) and its uridylylated form (reaction 2) of glutamine synthetase. The conditions of the assay were developed such that the appearance of enzyme activity was due entirely to ATase or P_{II}-stimulated formation of active subunits of glutamine synthetase. These assays are sufficiently sensitive to be used on crude extracts as well as homogenous preparations. We are currently isolating the ATase, P_{II} and P_{II}-D from both E. coli and P. putida.

Significance to Biomedical Research:

The mechanism of enzyme regulation through feedback inhibition and protein modification is the basis of a number of metabolic diseases in which control of metabolic pathways is achieved. Because of the key role in bacterial nitrogen metabolism, glutamine synthetase serves as a unique model to elucidate principles of enzyme and metabolism regulation.

Proposed Course of Research:

We will continued to develop a purification scheme for P. putida Atase, P_{II}, and $P_{II}D$. Homogenous preparations will allow further detailed and quantitative studies to be carried out on the involved cascade control system of glutamine synthetase.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00205-21 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Role of Selenium in Anaerobic Electron Transport and Methane Biosynthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thressa C. Stadtman	Chief, Section on Intermed. Metab. & Bioenergetics	LB	NHLI
OTHER:	Joyce E. Cone	Staff Fellow (see indiv. report)	LB	NHLI
	Rafael Martin	Visiting Fellow (Spanish fellowship July to Nov., Visiting Fellow since Nov. 24, 1975 - see indiv. report)	LB	NHLI
	Belinda Seto	Staff Fellow (see indiv. report)	LB	NHLI
	Jay B. Jones	Technical assistant and predoctoral student G.W. Univ. (see individual report)	LB	NHLI
	Joe N. Davis	Research assistant (in charge of anaerobic laboratory, amino acid analyzers, etc. - see individual report)	LB	NHLI

COOPERATING UNITS (if any)

Blair Bowers, Ph. D., Laboratory of Cell Biology, NHLI, NIH.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3.1	0.9

SUMMARY OF WORK (200 words or less - underline keywords)

The selenium moiety of the glycine reductase selenoprotein was isolated from ^{75}Se-labeled protein after alkylation with several different alkylating agents and identified as a selenocysteine residue. This is the first instance in which an essential organoselenium compound in a protein has been identified. The formate dehydrogenase, as well as the glycine reductase of C. sticklandii, was shown to be a selenoprotein and thus two selenium-dependent enzymes produced by the same organism are available for comparative studies.

Studies on further purification and characterization of the glycine reductase enzyme system, the formate dehydrogenases and the methane forming system have been continued in order to further understand these anaerobic oxidation-reduction systems.

PHS-6040
(12-75)

Project Description:

(1) Anaerobic metabolism of certain amino acids with special reference to the role of selenium, molybdenum, quinones, flavins and non-heme iron proteins in the electron transfer and phosphorylation reactions involved.

(a) Structure and function of the selenoprotein component of glycine reductase and its interreaction with the other protein components of the enzyme complex. Mode of biosynthesis of the selenium-containing moiety, a selenocysteine residue of the selenoprotein.

(b) Purification and characterization of additional components of the glycine reductase complex in order to determine the mechanism of the reaction and the coupled phosphorylation process.

(2) Isolation and characterization of selenium-dependent formate dehydrogenases.

(a) To identify the selenium-containing moiety of the enzymes from Clostridium sticklandii and from Methanococcus vannielii.

(b) Comparison of the enzymes produced in cells grown with tungstate instead of molybdate; if the tungsten enzyme is more stable and/or more active, this could explain the stimulatory effect of tungsten on growth of M. vannielii.

(3) Mechanism of methane formation from acetate and formate and role of vitamin B_{12} in the process.

Major Findings:

(1-2) The selenium-containing moiety of the glycine reductase selenoprotein A was shown to be selenocysteine. It was isolated from acid hydrolysates and enzymic digests of the alkylated ^{75}Se-protein and characterized as its Se-carboxymethyl, Se-carboxyethyl, Se-aminoethyl and Se-carboxymethyl-N-dinitrophenyl derivatives. The corresponding derivatives prepared from authentic Se-cysteine served as reference standards for ion exchange and thin layer chromatography. A low concentration of selenite (0.5 μm) is sufficient for the formation of the glycine reductase selenoprotein and the Se-dependent formate dehydrogenase of C. sticklandii. Incorporation of about 50% of this added selenium into the cells indicates the highly specific and efficient nature of the processes involved in selenoprotein biosynthesis. As yet, no inactive precursor form of the selenoprotein has been detected. Studies on the end groups and on the composition of small ^{75}Se-selenocysteine peptides from selenoprotein A are carried out by Dr. Rafael Martin and should aid in understanding the biological role of this small, heat stable protein.

Release of the membrane-bound glycine reductase components by treatment with detergents (studies by Joyce Cone) facilitates isolation of the larger molecular weight enzymes of the system and appears to improve their yield.

It is essential to have these proteins available in pure form in order to study the mechanism of the reaction and the nature of the phosphorylated intermediate that gives rise to ATP.

The formate dehydrogenase of C. sticklandii is also membrane bound and is solubilized by detergents. Partially purified preparations of the clostridial enzyme resemble in many respects the formate dehydrogenase of M. vannielii. Radioactive selenocysteine has been identified in digests of alkylated crude preparations of formate dehydrogenase from ^{75}Se-labeled C. sticklandii cells. Since enzyme activity can be detected directly on polyacrylamide gels after electrophoresis, provided oxygen is rigorously excluded, pure samples of ^{75}Se-formate dehydrogenase will be subjected to the same procedure to ascertain whether the selenium moiety of the enzyme is indeed a selenocysteine residue.

(3) Although growth of M. vannielii and methane production from formate are markedly stimulated by selenium, the organism, nevertheless, does grow on formate in Se deficient media, even though it can produce little formate dehydrogenase under these conditions. A by-pass mechanism involving pyruvate as intermediate could theoretically allow the indirect conversion of formate to carbon dioxide. A competitive inhibitor of pyruvate oxidation, glyoxylate, is effective with the M. vannielii enzyme preparations and is useful in studies to assess the significance of pyruvate under the two sets of conditions.

Proposed Course of Research:

(1) Further characterization of the oxidized and reduced forms of the glycine reductase selenoprotein with special reference to the composition of the active site and the biochemical role of the protein.

(2) Mode of biosynthesis of the selenocysteine moiety of the glycine reductase selenoprotein.

(3) Identification of the selenium moiety of the formate dehydrogenases and biochemistry of its synthesis and role.

(4) Reconstitution of the glycine reductase system from purified protein components; identification of partial reactions as a means of determining mechanism of the reduction and concomitant phosphorylation.

(5) Nature of early enzyme-bound intermediates in the reduction of carbon dioxide to methane and cofactors involved.

Publications:

Jay B. Jones and T.C. Stadtman. Methanococcus vannielii: Growth and Metabolism of Formate. In Symposium on "Microbial Production and Utilization of Gases." Akademie der Wissenschaften, Göttingen, Germany (In Press).

Joyce E. Cone, Rafael Martin del Rio, Joe Nathan Davis and Thressa C. Stadtman. Chemical Characterization of the Selenoprotein Component of Clostridial Glycine Reductase: Identification of Selenocysteine as the Organoselenium Moiety. Proc. Nat. Acad. Sci. (In Press).

Belinda Seto and Thressa C. Stadtman. Purification and Properties of Proline Reductase from Clostridium sticklandii. J. Biol. Chem. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00206-17 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Stereochemical Studies of Enzymatic Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lin Tsai	Chemist	LB NHLI
OTHER:	E. Caveney	Chemist	LB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism & Bioenergetics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The rearrangement catalyzed by a coenzyme B_{12} dependent enzyme, α-MG mutase, and an isomerase was studied with ^{14}C- and $^{3}H,^{14}C$-substrate. Substantial loss of tritium was observed in the conversion of α-methyleneglutarate to dimethylmaleate. This was probably caused by the presence of high concentration of the isomerase in the crude enzyme preparation.

(2) A cis and trans mixture of 2-amino-5-hydroxyhexanoic lactone was prepared. Tentative assignment of chemical shifts of all the proton resonance in both isomers was made with the aid of the spectra of two deuterated analogs; but some of the coupling constants were anomalous. No distinction between the cis and trans isomers could be made on the basis of these data.

PHS-6040
(12-75)

Project Description:

Objective 1: For the study of the steric course of α-MG mutase reaction, it is necessary to establish conclusively the inter- or intramolecular nature of the rearrangement.

Major Findings:

(1) A procedure was developed for the isolation and purification of the product, dimethylmaleate, from the reaction mixture without the need of adding a carrier. The specific radioactivity of the product thus obtained could be compared directly with that of the substrate. This procedure was greatly facilitated by the observation that dimethylmaleic anhydride in hydrocarbon solvent exhibited a prominent maximum at 244 nm ($\varepsilon = 5.1 \times 10^3$). This unusual spectral property served as a convenient means for determination of concentration of the product. Typically, a partially purified enzyme preparation could convert ^{14}C-α-methyleneglutarate (sp. act: 220 cpm/μmole) to ^{14}C-dimethylmaleic anhydride (sp. act : 230 cpm/μmole) in about 8-15% yield depending on enzyme concentration. Unfortunately, preliminary experiments using 3-^{3}H,2'-^{14}C-α-methyleneglutarate as substrate showed substantial loss of ^{3}H in the product. Since it has been shown that in the mutase the proton is transferred via B_{12}-coenzyme, the loss of tritium in these experiments could have occurred only in the isomerase step.

(2) Degradation of dimethylmaleic anhydride to acetate was accomplished by ozonization at pH 10 and 0°, followed by decomposition of the ozonide by alkaline H_2O_2 at 70°. The specific radioactivity of acetate was found to be half of that of the dimethylmaleate, thus confirming that all the ^{14}C-label in dimethylmaleate resided in the methyl groups which were derived from the ^{14}C label in the methylene group of the substrate.

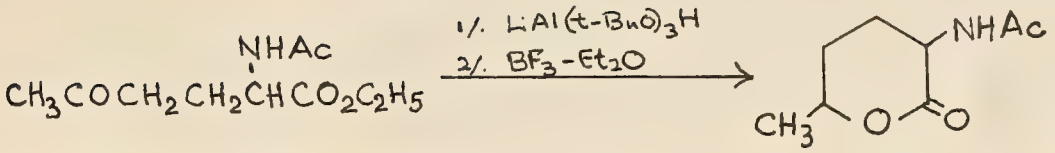
Proposed Course of Action:

To study the conditions for the mutase-isomerase reaction so as to minimize the loss of tritium in the process.

Objective 2: To study the chemical and spectroscopic properties of 2-amino-5-hydroxy-hexanoic acid.

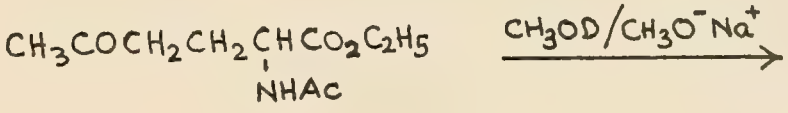
Major Findings:

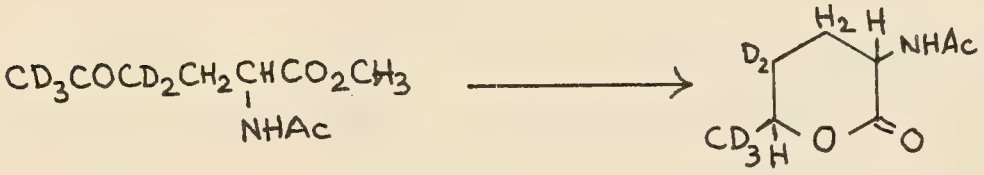
An approach to determine the relative configuration of the diasteroisomers of 2-amino-5-hydroxy-hexanoic acid is to convert it to a lactone whose NMR spectrum may reveal the cis and trans relationship of the two substituents. The lactone was prepared as follows:



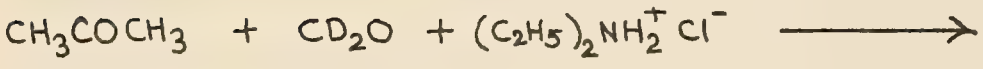
This procedure produced an isomeric mixture with one of them in slight excess, but attempts to separate these isomers were unrewarding. The NMR spectrum of the isomeric lactones was too complex for analysis. It was hoped that by replacing some of the hydrogen atoms with deuterium might simplify the spectrum. The two deuterated compounds, (A) and (B), were prepared as follows:

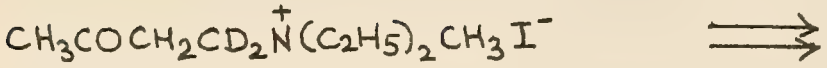
(A)

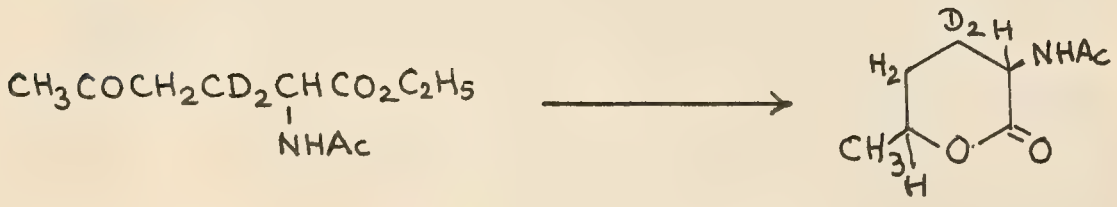




(B)







From the NMR spectra of both deuterated compounds, a tentative assignment of the chemical shifts of all the protons in both isomers could be made. However, some of the vicinal coupling constants from first-order approximation were anomalous, therefore, it was not possible to distinguish the cis and trans isomers on the basis of these data. This difficulty is probably due to some unusual feature of the conformation of six-membered lactones.

Proposed Course of Action:

To try to separate the isomeric lactones, and to modify the substituents so as to favor one conformation over another.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00207-02 LB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization of a bacterial selenoprotein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joyce E. Cone	Staff Fellow	LB NHLI
OTHER:	Thressa C. Stadtman	Chief, Section on Intermed. Metab. & Bioenergetics	LB NHLI
	Rafael Martin	Visiting Fellow	LB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

The selenium protein required for glycine reduction in Clostridium sticklandii has been characterized with regard to its fundamental physical and chemical properties. The selenium-containing moiety was identified as selenocysteine based upon the isolation of Se-aminoethyl-selenocysteine from the protein previously alkylated with ethylene imine. Purification of the additional enzymatic components of the glycine reductase system is being pursued in order to understand the function of the selenium protein in the overall reaction.

PHS-6040
(12-75)

Project Description:

Objectives: Chemical characterization of the selenium protein required for glycine reduction in Clostridium sticklandii; purification of additional enzyme and electron transport components required for the overall reaction.

Major Findings:

The fundamental molecular properties of the selenium protein have been determined with regard to molecular weight, amino acid composition, and identity of selenocysteine as the selenium-containing moiety of the protein. The selenium-containing residue was identified as Se-aminoethyl-selenocysteine after alkylation of the reduced protein with ethylene imine, followed by enzymatic digestion with trypsin and carboxypeptidase. After digestion of the aminoethylated protein with trypsin alone, a dipeptide containing Se-aminoethyl-selenocysteine at the carboxyterminal was produced. Titration of the reduced selenoprotein with 5,5'-dithiobis -2-nitrobenzoic acid (DNTB) gave three sulfhydryl equivalents per mole of protein (12,000 minimum molecular weight), consistent with the compositional analysis of two residues of cysteine and one residue of selenocysteine.

Partial purification of the additional enzymatic components required for glycine reduction has been accomplished through the use of detergents to solubilize and stabilize these proteins normally associated with the plasma membrane.

Proposed Course of Research:

Further purification of the enzyme and electron transport components required for the overall glycine reduction reaction will be pursued.

Publications:

Joyce E. Cone, Rafael Martin del Rio, Joe Nathan Davis and Thressa C. Stadtman. Chemical Characterization of the Selenoprotein Component of Clostridial Glycine Reductase: Identification of Selenocysteine as the Organoselenium Moiety. Proc. Nat. Acad. Sci. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00208-03 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Involvement of Pyruvate in the Reaction Mechanism of Proline Reductase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Belinda Seto	Staff Fellow	LB	NHLI
OTHER:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism & Bioenergetics	LB	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism & Bioenergetics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The major findings suggest that there is one pyruvate linked covalently to the N-terminal of each of the ten subunits of proline reductase. The pyruvate cofactors deriving from serine are essential for enzyme catalysis. Furthermore, an N-terminal peptide containing the pyruvate cofactor can be released by mild alkaline hydrolysis, suggesting an ester linkage.

PHS-6040
(12-75)

Project Description: Project No. ZO1 HL 00208-03 LB

Objectives: (1) To determine the stoichiometry of the pyruvate group and the nature of its linkage to proline reductase (2) to study the origin of pyruvate in proline reductase biosynthesis.

Major Findings:

(1) Proline reductase was inactivated by borohydride reduction or treatment with hydroxylamine and phenylhydrazine, suggesting the presence of an essential carbonyl cofactor. This cofactor was shown to be pyruvate, as reported earlier by Hodgins and Abeles.

(2) After reduction of the purified proline reductase with NaB^3H_4, 6N HCl hydrolysis liberates 10 moles [^{3}H]lactate per mole of enzyme. This suggests that each of the ten subunits of proline reductase contains a pyruvate prosthetic group. Furthermore, since no free N-terminal group was detected by dansylation or by digestion with aminopeptidase M, the N-terminal position of each subunit may be blocked by a pyruvate moiety.

(3) A pyruvate containing peptide of approximately 6000-7000 daltons is liberated from the protein by hydrolysis with 0.1 N NaOH at 100° for 5 min. The alkaline lability of the linkage between this peptide and proline reductase suggests that an ester bond may be involved.

(4) Proline reductase isolated from C. sticklandii culture supplemented [1-^{14}C]DL-serine was found to incorporate radioactivity into pyruvate and the serine residues. After reduction of proline reductase with $NaBH_4$, preliminary data indicated that the specific radioactivity of lactate was similar to that of the serine residues. It thus suggests that the pyruvate cofactors of proline reductase originate from serine and does not equilibrate with an endogenous pyruvate pool. Furthermore, the incorporation of pyruvate cofactor into proline reductase is most likely an indirect one through post-translational modification of serine.

Publications:

1. Belinda Seto and Thressa C. Stadtman: Purification and Properties of Proline Reductase from Clostridium sticklandii. J. Biol. Chem. in press.

2. Belinda Seto: Characterization of Enzyme-bound Pyruvate. Fed. Proc. 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00209-06 LB

PERIOD COVERED July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Menadione-Dependent-p-Nitrophenyl Phosphatase of Clostridium sticklandii

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. N. Davis	Biochemist	LB NHLI
OTHER:	T. C. Stadtman	Chief, Section on Intermed. Metab. & Bioenergetics	LB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism & Bioenergetics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7		0.7

SUMMARY OF WORK (200 words or less - underline keywords)

Continuing studies on the structure and properties of the dithiol and menadione-dependent p-nitrophenyl phosphatase of Clostridium sticklandii established that there are two cysteine residues per mole of protein. These presumably are maintained in the reduced form by the added dithiol during catalysis.

A crude lipid fraction extracted from C. sticklandii cells with an isooctane-isopropanol mixture contained a substance(s) that replaced menadione as activator of the enzyme. This active material presumably is the natural quinone required for activity of the phosphatase and experiments are in progress to characterize it further.

PHS-6040
(12-75)

Project Description:

Objectives: (1) To identify the natural substrate of the phosphatase enzyme. (2) To determine the precise biological role of this unusual quinone-dependent phosphatase. (3) To determine the composition and some physical properties of the enzyme protein.

Major Findings:

(1) Isolation of a naturally occurring quinone was attempted by extraction of both packed cells and cell suspensions with isoctane-isopropanol (3:1). The extracted quinone-like material was capable of replacing menadione as an activator. Reduction of the extracted material with KBH_4 increased its stimulatory effect.

(2) The pure phosphatase protein was reduced and carboxymethylated in order to determine the number of cysteines in the protein. Amino acid analysis indicated the presence of two carboxymethylated cysteine residues per mole of enzyme, based on a molecular weight of 30,000. This number is in agreement with the number of thiol groups found by titration of the pure protein with Ellman's reagent. DTNB titration indicated two thio groups in the enzyme. Experiments to quantitate the cysteine moieties of the protein as cysteic acid residues following oxidation with performic acid are in progress.

(3) The pure protein was hydrolyzed with Methanesulfonic acid in the presence of added 3-(2-aminoethyl) indole and aliquots of the hydrolysate were used for Tryptophan analysis on the (short) basic column of the amino acid analyzer. No tryptophan was detected although fluorescence spectra of the protein had indicated the presence of typtophan. Quanitation by the spectroscopic method of H. Edelhock suggests the presence of one tryptophan in addition to the two tyrosine residues determined by amino acid analysis. This quanitation must be rechecked spectroscopically, and additional amino acid analyses using larger amounts of protein will be performed in order to resolve this point.

Proposed Course of Action: Attempts will be continued to identify the natural phosphate ester substrate of the enzyme by (1) preparation of P^{32} labeled cell membrane protein which can be tested as substrates. (2) Continue to purify the extracted quinone and, after purification, to prepare the phosphate ester of same. (3) Phosphorylate the glycine reductase selenoprotein chemically and test as a possible substrate. (4) Chemically phosphorylate the other protein components of the glycine reductase complex and test as substrates.

Publications:

Cone, J. E., Martin del Rio, R., Davis, J. N., and Stadtman, T. C.: Chemical Characterization of the Selenoprotein Component of Clostridial Glycine Reductase: Identification of Selenocystein as the Organoselenium Moiety. PNAS (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00216-01 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Physiological Role of Selenium in Protein A of Glycine Reductase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rafael Martin	Visiting Fellow	LB NHLI
OTHER:	T. C. Stadtman	Chief, Section on Intermed. Metab. & Bioenergetics	LB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism & Bioenergetics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

End group analysis of protein A identified asparagine as the carboxy-terminal amino acid. No free amino terminal has been found, indicating that it is blocked. The selenium moiety of protein A has been identified as selenocysteine. It is the first essential selenium compound in a protein to be identified.

In preliminary studies it was shown that cyanogen bromide cleavage produces a peptide containing selenocysteine. This will be isolated in larger amounts and sequenced so that the neighboring groups may be determined. Experiments will be conducted to ascertain if the selenium is introduced into protein A as selenocysteine during protein synthesis or if it is added after the protein has been synthesized. The physiological role of selenium in the reaction that reduces glycine to acetate will also be investigated.

PHS-6040
(12-75)

radioactive compounds were isolated. If ^{75}Se existed in the protein as an analog of a sulfur compound, it might be expected to occur as a selenomethionine or selenocysteine residue. The latter possibility could explain why a radioactive amino acid was not found in the carboxypeptidase digest of the native protein, since selenocysteine is a very unstable compound. To test this possibility, protein A was reduced carefully with borohydride and alkylated with iodoacetic acid. Digestion of this modified protein for 72 hours with carboxypeptidase also released more than 70% of the radioactivity from the protein but, in this case, analysis of the amino acids in the digest on an automatic amino acid analyzer showed a peak tailing aspartic acid in the position where synthetic carboxymethyl Se-cysteine is eluted. In a parallel sample, 95% of the ^{75}Se in the digest eluted also in this position. Fractions collected from this peak were analyzed by thin layer chromatography and the radioactivity cochromatographed with synthetic carboxymethylselenocysteine. The conclusion is that selenocysteine is the selenium containing moiety in protein A.

Proposed Course of Action:

Two main questions remain to be investigated. How is the selenium (from selenite) incorporated into the protein in the form of selenocysteine? What is the physiological meaning of selenium and protein A in the glycine reductase system? These two questions are the proposed course of work for the next project.

Publications:

Joyce E. Cone, Rafael Martin del Rio, Joe Nathan Davis and Thressa C. Stadtman. Chemical Characterization of the Selenoprotein Component of Clostridial Glycine Reductase: Identification of Selenocysteine as the Organoselenium Moiety. Proc. Nat. Acad. Sci. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00218-01 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Formate Dehydrogenase from Clostridium sticklandii and Methanococcus vannielii.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jay B. Jones	Microbiologist	LB NHLI
OTHER:	T. C. Stadtman	Chief, Section on Intermed. Metab. & Bioenergetics	LB NHLI

COOPERATING UNITS (if any)

Dr. Blair Bowers, Laboratory of Cell Biology, NHLI, NIH.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism & Bioenergetics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

Formate dehydrogenase has been partially purified from two strictly anaerobic bacteria, Clostridium sticklandii and Methanococcus vannielii. The enzyme from M. vannielii is soluble, whereas the enzyme in C. sticklandii is membrane bound and is solubilized from the pellet fraction of whole cell sonicates by various ionic and non-ionic detergents, such as SDS and triton X-100. Activity stains on disc gels show multiple formate dehydrogenase bands from both sources. The enzymes from both organisms are similar in that they have a temperature optimum at 65°C, are extremely oxygen labile, are inhibited by cyanide, and have a bimodal pH optimum.

PHS-6040
(12-75)

Project Description:

Objectives: Formate dehydrogenases from several sources including E. coli and some clostridial species have been shown to be selenoenzymes that in addition contain iron and molybdenum. Although these trace elements presumably participate in the oxidation-reduction process, their precise catalytic roles are largely unknown. The formate dehydrogenases from the two strictly anaerobic bacteria, M. vannielii and C. sticklandii, are similar in properties and studies designed to characterize these interesting catalysts in more detail are underway. M. vannielii, which produces high levels of formate dehydrogenase, grows in a simple mineral salts medium with formate as sole carbon source. The levels of trace elements in this medium can easily be varied for studies of their effects on activity of the enzyme. C. sticklandii produces both a selenium-dependent formate dehydrogenase and the selenoprotein of the glycine reductase system and thus provides an excellent material for comparative studies on the biosynthesis and function of the two selenoenzymes.

Major Findings:

Large scale growth of M. vannielii has been hampered in the past by the fragility of the organism to mechanical shearing. The basis for this sensitivity resides in the lack of a stable cell wall. Culture techniques have been adapted so that fermentor yields are increased. Now that large amounts of the methane organism are easily obtainable, studies on the components responsible for oxidation of formate to CO_2 and its subsequent reduction to CH_4 are more practical.

(I) Morphology of M. vannielii: (electron microscopy studies carried out in collaboration with Dr. Blair Bowers) The marked fragility of M. vannielii cells to mechanical stress, the ability of the organism to grow in the presence of high levels of antibiotics whose mode of action is to block cell wall synthesis and the almost instantaneous lysis of the cells by low levels of detergents suggested that M. vannielii lacks a cell wall. However, electron micrographs of freeze fracture replicas show that a cell wall is present. A low peptidoglycan content may account for the atypical properties of the wall structure. Electron micrographs also show that M. vannielii possesses two to four polar tufts of flagella.

(II) Formate dehydrogenase from M. vannielii: The partially purified formate dehydrogenase from M. vannielii exhibits two catalytically active bands when subjected to electrophoresis on disc gels. These two forms show identical pH and temperature optima as well as sensitivity to cyanide and oxygen. The substrate partially protects both forms of the enzyme from inactivation by oxygen.

(III) Formate dehydrogenase from C. sticklandii: The membrane bound formate dehydrogenase is solubilized from the pellet fraction of whole cell sonicates by various ionic and non ionic detergents such as SDS and triton

X-100. Growth of the organism in the presence of 1.5% rather than 0.15% formate causes a marked increase in enzyme activity. The clostridial enzyme, like that from M. vannielii, is oxygen labile, has a bimodal pH optimum, is inhibited by cyanide, and has a temperature optimum of 65°C. Activity stains on disc gels also show multiple formate dehydrogenase bands.

Proposed Course of Action:

Purification of the formate dehydrogenases will be continued in order to obtain material for detailed study of structure and function. The possibility that selenium occurs in the form of selenocysteine (as in glycine reductase protein A) will be explored by subjecting reduced and alkylated ^{75}Se-formate dehydrogenase to hydrolysis and subsequent amino acid analysis. ^{75}Se labeled peptide fragments from both bacterial sources will be tested for homology and compared to protein A peptide fragments. The manner in which selenoprotein is synthesized and degraded will be explored by searching for ^{75}Se labeled intermediates in vivo and in vitro.

Incorporation of Mo, W, and Fe into formate dehydrogenase will be tested using isotope and chemical assay methods on purified protein. Ratios of molybdate to tungsten incorporation will be measured in enzyme preparations from cells grown in media of differing compositions of these metals to test if tungsten is preferentially incorporated to produce more stable enzyme. Subunit composition will be determined to elucidate the site of incorporation of these various elements.

Publications:

Jay B. Jones and T.C. Stadtman. Methanococcus vannielii: Growth and Metabolism of Formate. In Symposium on "Microbial Production and Utilization of Gases." Akademie der Wissenschaften, Göttingen, Germany (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00204-09 LB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Protein Structure: Enzyme Action and Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ann Ginsburg, Head, Section on Protein Chemistry	LB	NHLI
OTHER:	Donald M. Powers, Staff Fellow	LB	NHLI
	Andrew Shrake, Staff Fellow	LB	NHLI

COOPERATING UNITS (if any)
R. Park, Section on Enzymes, LB NHLI

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.46	3.16	.3

SUMMARY OF WORK (200 words or less - underline keywords)

Research in this section consists of studies on the physical and chemical properties of proteins of biological interest and the roles of ligand binding and protein-protein interactions in enzyme catalysis and regulation.

1. Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli have been studied by microcalorimetry and equilibrium dialysis techniques.

2. The interaction of the glutamine synthetase adenylyltransferase with various glutamine analogs was investigated. This enzyme, which catalyzes the adenylylation and deadenylylation of glutamine synthetase, has allosteric sites for both glutamine and the regulatory P_{II} protein.

3. The glutamyl-tRNA synthetase from E. coli has been purified to homogeneity and found to be activated by a protein of ~74,000 mol. wt (74K protein). The separation of these two proteins recently was achieved by preparative gel electrophoresis. A possible regulation of the glutamyl-tRNA synthetase (63,000 mol. wt.) by the 74K protein currently is being investigated.

PHS-6040
(12-75)

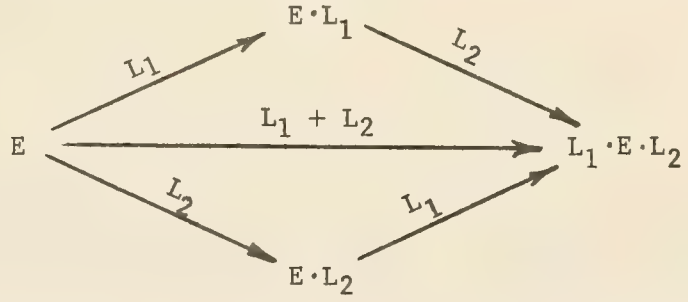
Project Description:

Objectives: (1) To study the physical and chemical properties of glutamine synthetase from Escherichia coli, particularly with respect to the correlation of the structure and catalytic function of this enzyme. (2) To study conformation and stabilization changes of a protein macromolecule effected through the specific binding of small molecules, and the relationship of such effects to enzyme catalysis and regulation. (3) Ultracentrifugation studies to determine macromolecular properties of biologically important proteins. (4) To study the ATP-glutamine synthetase adenylyltransferase from E. coli, with emphasis on the mechanism of action and the physical structure of this enzyme.(5) To isolate the glutamyl-tRNA synthetase from E. coli in order to investigate the structure and regulation of this enzyme.

Major Findings:

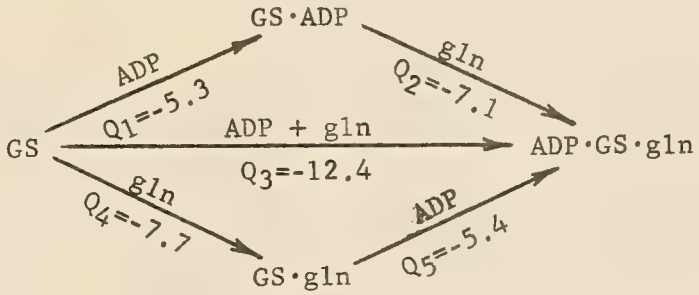
1. Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli. (Investigators: A. Shrake, D. M. Powers, and A. Ginsburg). This study is an extension of previous investigations on the role of various effectors in enzyme catalysis. Glutamine synthetase, a strictly regulated enzyme in E. coli, is a dodecamer with twelve catalytic sites. Heats of binding of substrates and inhibitors to the unadenylylated enzyme have been measured at 30° in a batch-type microcalorimeter. These calorimetrically determined heats have provided information on the separateness of binding sites and have given accurate thermodynamic constants for the binding of low affinity ligands.

The separateness of binding sites of ligands L_1 and L_2 on an enzyme E can be demonstrated by measuring the overall heat of binding of both ligands for any two of the three possible paths in the scheme below. If the same



buffer is used and if the final ligand concentrations are held constant, equal overall heats for any two paths suggest separate binding sites for L_1 and L_2. This is true for saturating and subsaturating ligand concentrations, for non-interacting and interacting effectors (those exhibiting synergism or antagonism), and for the absence or presence of concomitant proton uptake or release. Unequal overall heats for the different paths may indicate competition at a common binding site, mutually exclusive binding of L_1 and L_2, or slow kinetic effects that require additional experimental data for interpretation.

Measurements of the heats of binding of two substrates, ADP and L-glutamine, to glutamine synthetase (GS) demonstrate the equality of overall heat for the three paths. The heats were measured in 0.050 Hepes, 0.10 M KCl, and 0.001 M $MnCl_2$ at pH 7.1 at 30° with a final free concentration of 0.1 mM for ADP and 30 mM for glutamine. Calorimetric measurements (corrected for heats of dilution) are shown for the different reactions, where the heat of ligand binding (Q) is expressed in kcal per mole of enzyme subunits.



Within experimental error, the overall heats in the top path ($Q_1 + Q_2 = -12.4$), the middle path ($Q_3 = -12.4$), and the bottom path ($Q_4 + Q_5 = -13.1$) are equal. In this study, glutamine is not saturating, ADP is saturating throughout and enhances the affinity of the enzyme for glutamine thereby changing the degree of saturation of the enzyme with glutamine, and a small proton uptake occurs in the overall reaction.

Calorimetric measurements have demonstrated separate enzyme binding sites for the feedback inhibitors AMP and L-tryptophan and for the substrate L-glutamate and the inhibitor L-alanine (work by R. Park). However, calorimetry could not be used to show separate sites for AMP and ADP even though equilibrium binding measurements with [^{14}C]ADP and [^{32}P]AMP showed that both compounds bind simultaneously to each of the enzyme subunits. Possibly, AMP does not appreciably bind to the enzyme-ADP complex during the time of the calorimeter experiment since the rate of binding of AMP in the presence of ADP during equilibrium dialysis measurements was extremely slow. However, calorimetry has provided indirect evidence that the enzyme subunit sites for binding the inhibitor AMP and the substrate ADP are different. The use of buffers at pH 7.1 with quite different heats of protonation (Tris, imidazole, or Hepes) showed that the binding of AMP caused a net proton uptake of 0.57 equiv per mole of subunit, with $\Delta H' = -10$ kcal/mole after correcting for the proton uptake. In contrast, the binding of ADP ($\Delta H' = -5.2$ kcal/mole) causes little or no proton uptake.

The binding of glutamine to glutamine synthetase also was measured by calorimetry. Values for K_D' and $\Delta H'$ were obtained through fitting data from the thermal saturation curves to the Scatchard equation using a least squares fitting procedure, in which the experimental points were weighted according to the estimated error in both Q and Q/(S) where Q is the heat of binding in kcal/mole and (S) is the free substrate concentration. We assumed an error of 0.17 mcal in the observed heat and no error in substrate concentration. For the binding of glutamine to unadenylylated glutamine synthetase

(in buffers at pH 7.1 containing 0.10 M KCl and 1 mM $MnCl_2$) in the presence and absence of ADP, K_D' values for glutamine are 1.7 and 7.3 mM and corresponding $\Delta H'$ values are -7.4 and -9.7 kcal/mole, respectively. The synergistic effect between glutamine and ADP is reflected in these data. The thermal saturation curve for the binding of glutamine to completely adenylylated glutamine synthetase in the absence of ADP gave K_D' = 5.4 mM. This was an unexpected result since unpublished data indicate that the K_m' value for glutamine in the γ-glutamyl transfer reaction is increased by adenylylation of the enzyme. The binding of Mn^{2+} and other effectors to the adenylylated enzyme currently is being investigated.

2. Studies on the ATP:glutamine synthetase adenylyltransferase from E. coli. (Investigator: A. Ginsburg). Regulation of glutamine synthetase in E. coli is mediated by adenylylation and deadenylylation, a covalent modification of glutamine synthetase catalyzed by adenylyltransferase and also involving a small regulatory protein. Some studies on the chemical and regulatory properties of the adenylyltransferase are continuing. The enzyme has allosteric sites for L-glutamine and for the regulatory P_{II} protein. A glutamine analog, albizzin (L-2-amino-3-ureidopropionic acid), is about one-half as effective at a saturating concentration as is glutamine in activating the enzyme in the adenylylation reaction. The enzyme has a lower affinity for albizzin ($K_{activation}$ = 16 mM) than for glutamine ($K_{activation}$ = 0.2 mM). Adenylyltransferase was inactive with methionine sulfoximine (another glutamine analog); this is probably related to the fact that this compound inactivates glutamine synthetase. A covalent modification of the glutamine binding site was attempted by reacting adenylyltransferase with chloroketone (2-amino-4-oxo-5-chloropentanoic acid, a glutamine analog described by E. Kedouri, P. M. Anderson, and A. Meister, Biochemistry 5, 3552, 1966). This treatment did not produce an activation of the enzyme in catalyzing adenylylation or a loss in the ability of glutamine (or albizzin) to activate adenylyltransferase in adenylylating glutamine synthetase. These results suggest that chloroketone does not alkylate the allosteric glutamine site of adenylyltransferase.

3. Glutamyl-tRNA synthetase from E. coli. (Principal Investigator: D. M. Powers). Glutamyl-tRNA synthetase (GluRS) from E. coli has been reported to exist as an $\alpha\beta$ complex of 102,000 molecular weight with a catalytic subunit of 56,000 molecular weight and a regulatory subunit of 46,000 molecular weight (J. Lapointe and D. Söll, J. Biol. Chem. 247, 4966, 1972). Our investigation thus far has failed to confirm the existence of an oligomeric structure for GluRS. Analysis of GluRS in crude extracts of E. coli grown under varying nutritional conditions, as well as of purified GluRS, yielded a molecular weight of 63,000 by the polyacrylamide gel electrophoresis (PAGE) technique. Our results do not exclude the possibility that GluRS exists in vivo in a weakly associated complex, which may not be detectable by our methods.

In order to continue our studies on the GluRS, the scale of the Lapointe Söll purification procedure was increased ten-fold. A protease inhibitor (phenylmethylsulfonylfluoride) was routinely included in all of our purification

buffers, because the two peaks of GluRS activity which eluted from the hydroxyapatite column step during previous preparations were reminiscent of other cases of limited proteolysis. With the protease inhibitor present, a single peak of GluRS activity chromatographed on hydroxyapatite; however, the enzyme obtained was less than 40% pure. The remaining impurities proved very difficult to remove. After a large variety of additional steps, including affinity elution chromatography, ω-aminohexylagarose chromatography, gel filtration through Bio-gel P150 and Agarose 0.5m, and adsorption to an organomercurial Sepharose, as well as rechromatography on DEAE-cellulose and hydroxyapatite under different conditions, the enzyme was still only 60% pure. Five discrete bands were visible on analytical PAGE, with molecular weights of 46,000, 63,000 (GluRS), 74,000, 84,000 and 86,000.

A final purification of GluRS was achieved by two preparative PAGE (Bis-Tris, pH 7 system) runs. The first electrophoresis removed all but the 74,000 mol. wt. contaminant (74K protein); the second electrophoresis (with a longer and narrower gel column) resulted in two well-separated protein fractions which were homogeneous by analytical PAGE. The successful removal of the 74K protein from GluRS was accompanied by a precipitous drop in the specific activity of the GluRS. The recoveries of GluRS activity after the first and second electrophoresis were 85 and 15%, respectively. To determine whether the removal of the 74K protein in the second electrophoresis might be responsible for the loss in GluRS activity, aliquots of the GluRS and 74K protein fractions were combined and assayed for glutamyl-tRNA synthetase activity . There was a substantial recovery of GluRS activity that was proportional to the amount of the 74K protein included in the reaction mixtures. This result suggests that the 74K protein may have a role in regulating GluRS activity *in vivo**, and possibly provides an explanation for genetic data which show GluRS mapping in two widely separated regions of the *E. coli* chromosome (R.R.B. Russell and A. J. Pittard, J. Bacteriol. 108, 790, 1971). Studies are in progress to determine the conditions for attaining maximum stimulation of GluRS activity by recombination of the GluRS protein with the 74K protein and the influence of the 74K protein on the kinetic parameters of the GluRS-catalyzed acylation of tRNA and the ATP-pyrophosphate exchange reaction.

Significance to Bio-Medical Research

The regulation and control of enzymic activities *in vivo* is of fundamental importance in cellular metabolism. Through studies *in vitro*, these processes can be understood more fully. The study of structural changes that can be induced in a protein macromolecule are important in understanding cellular processes on a molecular basis.

*A stimulation of GluRS activity *in vitro* by acidic proteins (bovine serum albumin, *E. coli* adenylyltransferase and the regulatory P_{II} protein) has been observed previously.

Proposed Course of Research:

(1) To study conformational and stabilization changes of a protein macromolecule effected through the specific binding of small molecules, and the relationship of such effects to enzyme catalysis and regulation. Ultracentrifugation, microcalorimetry, spectral, fluorescence, equilibrium binding, and kinetic techniques will be used. In addition, a gel method of zone transport will be standardized for measuring affinity constants of interacting molecules.

(2) To study mutual interactions of divalent cations, substrates and inhibitors with glutamine synthetase from E. coli. Pyridoxylation of the subunit catalytic site will be tried to obtain information on the topography of substrate and inhibitor sites and on subunit interactions within the dodecamer.

(3) Physical and chemical studies of the E. coli ATP: glutamine synthetase adenylyltransferase will be continued.

(4) In collaboration with D. A. Zopf and V. Ginsburg (Section on Enzymes, Laboratory of Biochemistry, NIAMDD, NIH), to produce an agglutainating activity in a goat IgG antibody directed against a human Le^b blood group hapten (Lacto-N-Difucohexaose I) by a polymerization reaction involving intermolecular cross-linking with bifunctional imidoesters. A general technique for producing a strongly agglutinating antibody from IgG would be valuable clinically as a replacement for types of rare sera or sera that give only low positives in agglutination assays.

Publications:

Ginsburg, A., and Stadtman, E. R.: Glutamine Synthetase of Escherichia coli: Structure and Regulation. In Ebner, K. E. (Ed): Subunit Enzymes: Biochemistry and Function. New York, M. Dekker, Inc., 1975, pp. 43-84.

Hunt, J. B., Smyrniotis, P. Z., Ginsburg, A., and Stadtman, E. R.: Metal Ion Requirement by Glutamine Synthetase of Escherichia coli in Catalysis of γ-Glutamyl Transfer. Arch. Biochem. Biophys., 166: 102-124, 1975.

Zopf, D. A., Ginsburg, A., and Ginsburg, V.: Goat Antibody Directed Against a Human Le^b Blood Group Hapten, Lacto-N- Difucohexaose I. J. Immunology 115: 1525-1529, 1975.

Stadtman, E. R., Ciardi, J. E., Smyrniotis, P. Z., Segal, A., Ginsburg, A., and Adler, S. P.: Role of Adenylylated Glutamine Synthetase Enzymes and Uridylylated Regulatory Protein Enzymes in the Regulation of Glutamine Synthetase Activity in Escherichia coli. Isozymes II: Physiological Function, 1975, pp. 715-732.

Caban, C. E., and Ginsburg, A.: Glutamine Synthetase Adenylyltransferase from Escherichia coli: Purification, Physical and Chemical Properties. Biochemistry 15: pp. 1569-1580, 1976.

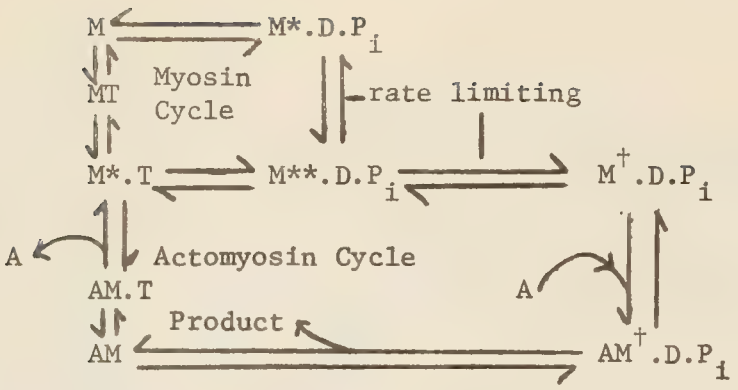
Annual Report of the
Laboratory of Cell Biology
National Heart and Lung Institute
July 1, 1975 through June 30, 1976

As in previous years, the research of the four Sections of the Laboratory of Cell Biology can be divided into the following seven areas: (1) the biochemistry of muscle contraction (2) the biochemistry and ultrastructure of cell motility (3) the structure, function and assembly of microtubules (4) the structural and functional interrelationships between plasma and intracellular membranes (5) the mechanisms of electron transport (6) multienzyme complexes involved in DNA biosynthesis, and (7) the structure and conformation of proteins. A selected sample of accomplishments this year will be reviewed in this summary. Before doing so, we note the retirement of Dr. Wayne Kielley, a member of the staff of this institute since 1951 and a major contributor to the fields of oxidative phosphorylation and muscle biochemistry.

Muscle Biochemistry: In previous years, investigators in this laboratory made the important discovery that myosin can exist in two states: a refractory state to which actin cannot bind and a non-refractory state which binds actin. It was proposed that the conversion of myosin from the refractory to the non-refractory state may be the rate-limiting event in the cyclical interaction of actin, myosin and ATP. Efforts are continuing to prove that this is the correct biochemical model and that it can explain the physiology of muscle contraction.

Because of the insolubility of actomyosin, the above studies were carried out with heavy meromyosin (HMM) and subfragment-1 (S-1), proteolytic degradation products of myosin that retain all of its specific properties and which form soluble complexes with actin. We have now been shown that the presteady state kinetics, as measured by the stopped-flow technique, of myosin-ATPase (in the absence of actin) are the same as for HMM and S-1. This provides further validation for concluding that data obtained for acto-HMM ATPase and actin-S-1 ATPase can be extrapolated to actomyosin ATPase.

Recent studies show that, in a single cycle of hydrolysis of ATP, acto-S-1 is dissociated by the ATP and reforms when the ATP is hydrolyzed. Importantly, the rate of reassociation of actin and S-1 in the single cycle equals the steady state rate of hydrolysis of ATP at all actin concentrations. This suggests that dissociation and reassociation occur each time an ATP molecule is hydrolyzed. This result requires that the rate-limiting step in the cycle occurs before reassociation, i.e. it is the conversion of refractory to non-refractory myosin as previously proposed. A change in fluorescence has been measured that is 10 times faster than the ATPase cycle. This may be a measure of the formation of refractory S-1 from the acto-S-1 complex. Taken altogether, these, and earlier, data can be integrated with the myosin-ATPase cycle proposed by others to explain the actomyosin-ATPase cycle as follows:



where M is myosin, AM is actomyosin, T is ATP, D is ADP, P_i is orthophosphate, and the asterisk and † represent different conformational states of the myosin.

In sum, actomyosin dissociates and reassociates each time a molecule of ATP is hydrolyzed. The dissociation of actomyosin by ATP is very rapid and its re-association is slow because of the slow conversion of refractory to non-refractory myosin.

This model fits the 1957 cross-bridge model for muscle contraction proposed by Huxley. The model required a slow formation of cross-bridge between actin and myosin and a rapid dissociation of cross-bridge following the work stroke. The slow step is now identified as the transition of refractory to non-refractory myosin. In the original Huxley model it was necessary to propose an "elastic" element in the myosin cross-bridge. In collaboration with Dr. Terrell Hill (NIAMDD) the Huxley model has been further modified to remove the elastic element by suggesting that the cross-bridge "rocks" around either of two stable angles (approximately 90° and 45°). The energy of ATP hydrolysis would be stored in the dissociation of actin and myosin with the "work step" of the cycle being the restoration of the strong actomyosin link as the cross-bridge "rocks" from an angle of 90° to the stable "rigor" angle of 45°. In the above scheme, there would be two forms of AM.D.P_i, one with a cross-bridge of 90° and one at 45°. Most of the physiological events of muscle contraction can be explained by this model which is derived from the kinetics of acto-HMM and acto-S-1 ATPase.

Biochemistry of Cell Motility: It is now known that cytoplasmic actin and myosin provide the molecular basis for many motile systems in all eukaryotic cells. Although actin has been identified as the major component of cytoplasmic microfilaments in more than 60 animal and plant cells, from amoeba to man, in only about 10 instances has the actin been isolated and partially characterized. The major reason for the paucity of information has been the difficulty of isolating cytoplasmic actin in good yield and, therefore, in sufficient quantity for biochemical studies.

We have now developed a new method, the critical step of which is chromatography on DEAE-cellulose, for the isolation of actin in 20% yield from *Acanthamoeba castellanii* where actin represents about 15% of the total cell protein. The new method is particularly useful when applied to cells such as platelets and erythrocytes in which actin is a relatively minor component. *Acanthamoeba* actin is now the best characterized cytoplasmic actin. Its amino acid composi-

tion is very similar to muscle actin including one residue of 3-methylhistidine. It also contains a residue of dimethyllysine which is unique to this actin. (The sequence of Acanthamoeba actin is presently being determined by Dr. Marshall Elzinga.) Acanthamoeba actin resembles muscle actin in having one mole of bound nucleotide per mole of actin monomer, in binding one mole of myosin ATPase head per mole of monomer, in its ability to activate the Mg-ATPase activity of muscle myosin and to inhibit the K-ATPase of muscle myosin. There are significant differences, however, between Acanthamoeba and muscle actin. Acanthamoeba actin is only one-third as effective in activating the Mg-ATPase of muscle myosin; although the maximal velocities at saturating actin concentrations are the same, it takes three times as much Acanthamoeba actin to reach half maximal velocity. Also, the critical concentration required for polymerization at 5° in 0.1M KCl (no Mg^{2+}) is 5 times greater for Acanthamoeba actin (0.5 mg/ml vs 0.1 mg/ml) and the rate of polymerization, under these conditions, is much slower for Acanthamoeba actin. Mixtures of Acanthamoeba and muscle actin can form co-polymers. Since 50% or more of cytoplasmic actin may be depolymerized and formation of polymerized actin may have functional significance it is important to study these properties of cytoplasmic actins in detail.

Under appropriate conditions (5 mM Mg^{2+}, 2 mM ATP) muscle tropomyosin binds to muscle actin in the molar ratio of 1:7 as measured by the binding of [^{125}I] tropomyosin and by the inhibition of actomyosin Mg-ATPase. Under these same conditions, binding of [^{125}I]tropomyosin to Acanthamoeba actin is too weak to detect and no inhibition of Acanthamoeba acto-HMM ATPase occurs. Tropomyosin does bind to Acanthamoeba acto-HMM in the absence of ATP and to Acanthamoeba actin in the presence of 80 mM KCl. In the latter case, tropomyosin activates rather than inhibits the acto-HMM Mg-ATPase in contrast to the effects on muscle acto-HMM. These very interesting differences between Acanthamoeba and muscle actin should help greatly in elucidating the poorly understood interactions between actin, tropomyosin, troponin and myosin in muscle as well as providing information about the cytoplasmic systems.

Several other laboratories have recently reported that appropriate cold extracts of cells (sea urchin, macrophage, fibroblast and Acanthamoeba) form solid gels (slowly in the cold and rapidly when warmed) that will later retract when warmed. This phenomenon may be a reflection of motile events in situ which may involve the formation and movement of gelled actin filaments. The in vitro phenomenon has been attributed by others to the interaction with F-actin filaments of one or more proteins including a high molecular weight (280,000) actin-binding protein and cytoplasmic myosins. The evidence is indirect and non-quantitative, however.

We have now begun to search for components in extracts of Acanthamoeba that can induce the gellation of actin filaments. Four proteins have been purified several hundred fold; each alone induces actin (2 mg/ml) to form solid gels when added in molar ratios of approximately 1:1000. In all cases the gellation is maximal at 30°. Interaction of the proteins with actin can be measured quantitatively by the increase in viscosity and by their inhibition of actin-activated HMM-ATPase. The four proteins have molecular weights of approximately 78,000, 64,000, 54,000 and 23,000; the first three may be dimers.

Microtubule Assembly

Cytoplasmic microtubules and mitotic spindles are polymers of a dimeric subunit of α-and β-tubulin (each a 55,000 dalton polypeptide) with which they are in dynamic equilibrium. Depolymerization can be induced by low temperature or high pressure *in situ* or with isolated microtubules. Microtubules also rapidly form and disappear *in vivo* but the natural regulatory mechanisms that control the state of polymerization of these important cellular organelles are unknown. Possible candidates for regulation include interactions with GTP that have been studied previously in this, and other, laboratories, phosphorylation of a serine residue, and a unique tyrosylation of the C-terminal glutamyl residue of tubulin. This latter reaction has been actively investigated in this Laboratory this year.

A tubulin-tyrosine ligase, detected in every rat tissue examined, has been purified about 40-fold (10% yield) from bovine brain. This enzyme is not present in single cell eukaryotic organisms. Preliminary evidence suggests that the enzyme is a dimer of about 150,000 daltons and that dimerization may be controlled by a small regulatory subunit. The ligase is specific for tubulin as the acceptor protein; even other proteins with a C-terminal glutamyl group are inactive. Dimeric tubulin is definitely a substrate but data are inconclusive on whether fully polymerized microtubules or partially polymerized rings and spirals can act as acceptors. (Rings and spirals are thought to be intermediates in polymerization of dimeric subunits to microtubules.) Although tubulin is the only acceptor protein, L-phenylalanine and L-DOPA can substitute (poorly) for L-tyrosine and L-tyr-L-tyr, L-glu-L-glu and tyramine are competitive inhibitors in the reaction. The only requirements other than L-tyrosine and tubulin are ATP, Mg^{2+} and K^{+}. In the presence of ADP and Pi (or arsenate) added tyrosine can be released from tyrosylated tubulin.

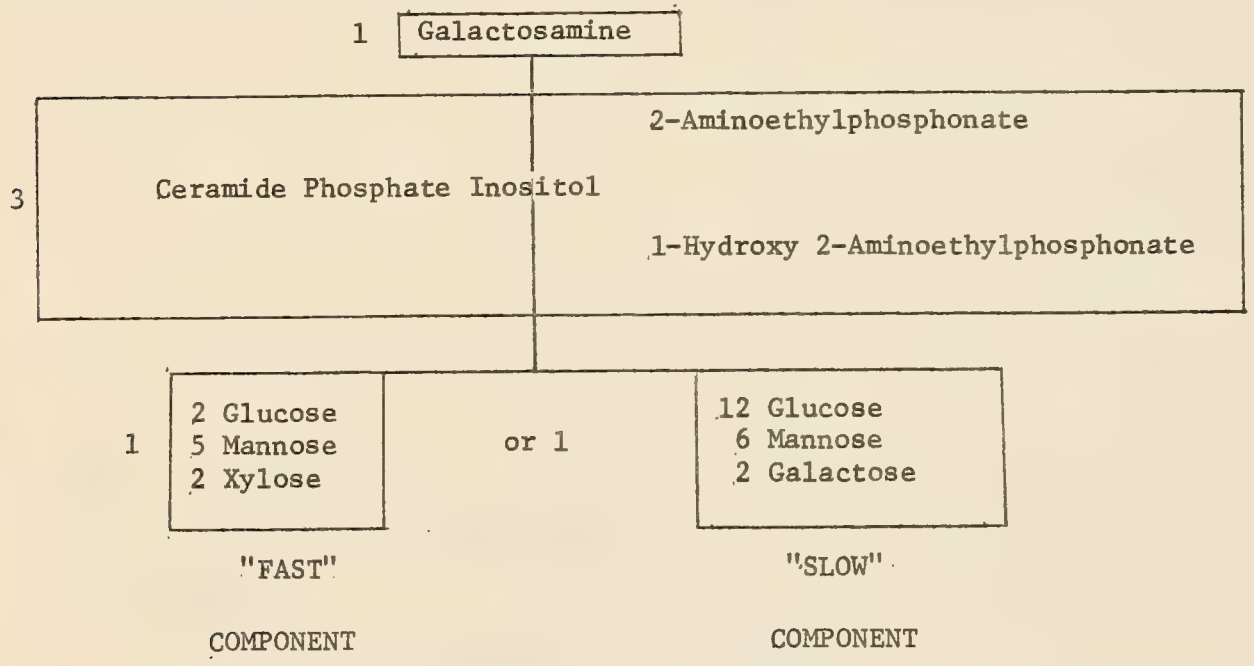
Although tyrosylated tubulin has been shown to be competent to polymerize, there is no evidence yet that tyrosylation is required for polymerization of tubulin dimer. In fact, available evidence suggests that native tubulin, as isolated, is not tyrosylated to a great extent. However, since only about 20% of α-tubulin can be tyrosylated in the reaction *in vitro*, we suspect that the terminal glutamyl residue of the native molecule may be blocked by an unknown compound of which tyrosine may be an analogue.

Plasma and Intracellular Membranes

Acanthamoeba castellanii continues to be a useful experimental model for studying the interrelationships and interactions of internal cell membranes and the plasma membrane in endocytic events. Through improved electron microscopic cytochemical techniques, we have detected three different acid phosphatase-positive vesicles in the amoeba: small golgi vesicles, large digestive vacuoles and mitochondrial-size vesicles of unknown origin and function. These latter vesicles resemble structures previously identified by others as peroxisomes but the presence of acid phosphatase is not to be expected in peroxisomes. From kinetic, cytochemical studies during phagocytosis of yeast, we conclude that acid phosphatase appears in the newly formed phagosomes by fusion directly with the small golgi vesicles and not by fusion of phagosomes with the large acid phosphatase-positive vesicles. Large acid phosphatase-

positive vacuoles do decrease during phagocytosis but probably because the golgi vesicles are fusing with phagosomes rather than with the "empty" vacuoles. The interrelationship among these membrane systems is also being studied by stereological and freeze-fracture electron microscopy.

In the course of this research we have characterized the chemical and enzymatic composition of the plasma membranes and endocytic membranes as reported previously. One of the more interesting constituents (about 30% of the membrane mass) is an unusual polymeric glycosphingolipid that seems to be exposed at both the external and cytoplasmic surfaces of the plasma membrane. We have now accounted for most of the constituents of this molecule, which is formed of two electrophoretically separable subunits ("fast" and "slow"), and can propose the following tentative working model for its structure:



Electron Transport: Coupled electron transport and oxidative phosphorylation are the major energy sources of aerobic cells. These are highly integrated membrane processes that will be completely understood only when the components are separately isolated, characterized and reconstituted. By sequential extraction with 0.4% and 2% deoxycholate, the succinoxidase activity (electron transport from succinate to 0_2) of E. coli membranes has been separated into two fractions, one enriched in succinate dehydrogenase, cytochrome B_1 and non-heme iron and the other enriched in cytochrome oxidase. Preliminary evidence suggests that it is possible to recombine these two non-membrane fractions and reconstitute succinoxidase activity by removing the deoxycholate by passage over a Sephadex column. An ultrafiltrate has been obtained from the cytochrome oxidase fraction that may contain as many as three factors that can stimulate the reconstitution.

DNA Biosynthesis: Previous work in this laboratory has led to the purification (40,000-fold) from E. coli of a complex of recBC and polymerase I that may be involved in DNA synthesis in vivo. Preliminary evidence now suggests that it

may be possible to synthesize this complex in vitro from purified recBC and polymerase I in the presence of DNA. The DNA can then be largely removed by the action of pancreatic DNase.

Protein Structure: Accepted dogma holds that completely reduced proteins contain no ordered structure. However, we have now observed that totally reduced and alkylated lysozyme still retains some of its original content of α-helix and has enhanced (2-3-fold) β-structure. Among other proteins studied (ribonuclease, papain, elastase, chymotrypsin and cytochrome c) some follow the pattern of lysozyme while others show reduced α-helix and reduced β-structure. In no case was all structure lost. These studies suggest that newly biosynthesized polypeptides may possess inherent structure that may play a significant role in directing the folding of the molecules into their final conformations.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00501-02 LCB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Acanthamoeba Actin: Isolation and Role in Cell Motility

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.J. Gordon	Staff Associate	LCB NHLI
	E.D. Korn	Chief, Lab. Cell Biol.	LCB NHLI
OTHER:	Y.-Z. Yang	Visiting Fellow	LCB NHLI
	E. Eisenberg	Head, Sec. Cell. Phys.	LCB NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.45	1.45	0

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed a new procedure employing chromatography on DEAE-cellulose for isolating actin in high yield from Acanthamoeba castellanii. We have found this actin to be similar to muscle actin in most of its biochemical properties, but with important quantitative differences in its polymerization behavior and in its interaction with myosin and tropomyosin. We plan to extend these investigations to other proteins (such as tubulin and spectrin) which may interact with the cytoplasmic actomyosin system and modulate its role in cell motility.

97

PHS-6040
(12-75)

Project Description:

Objectives: Our objective has been the isolation and biochemical characterization of Acanthamoeba actin. Using rabbit skeletal muscle as a reference for comparison, we have focused on those properties which might illuminate the respective roles of the two species of actin in cell motility and muscle contraction. Ultimately we would like to characterize the interaction of actin with other components of cytoplasmic actomyosin systems in order to understand the molecular mechanism of cell motility.

Methods Employed:

Chromatography on DEAE-cellulose and Sephadex has been employed in the purification of Acanthamoeba actin. ATPase activities have been measured by the pH stat method. Polymerization of actin has been assayed qualitatively by flow birefringence and quantitatively by viscosity and absorbance changes. Purity of actin preparations has been monitored by dodecyl sulfate gel electrophoresis.

Results:

A new procedure has been devised for isolating actin in high yield (15-20%) and purity (95%) from Acanthamoeba castellanii. Its unique feature is the employment of ion exchange chromatography on DEAE-cellulose as the initial purification step. This method avoids the major pitfall of other cytoplasmic actin purifications, including that of Weihing and Korn (Biochemistry 10, 590-600 (1971) for Acanthamoeba actin, which are based on the ability of actin to complex with exogenous or endogenous myosin in crude cell extracts. Since the bulk of cytoplasmic actin fails to form such complexes in most systems, the yield of these purification procedures is generally less than 2%. Because our method does not require actomyosin formation, one might expect it to be applicable to a wide variety of cell types, particularly those in which actin is only a minor component. Preliminary experiments in this laboratory with one such cell type, the erythrocyte, have been encouraging in this regard.

Having isolated Acanthamoeba actin in the quantity and purity required for our studies, we then examined several of its important biochemical properties. These include amino acid composition, bound nucleotide content, equilibrium and kinetics of polymerization under varying conditions, interaction with the rabbit skeletal muscle myosin derivatives heavy meromyosin (HMM) and subfragment-1 (S-1), and interaction with rabbit skeletal muscle tropomyosin (see Z01 HL 00412-01 LCB). In general, the properties were quite similar to those of muscle actin. The amino acid compositions of Acanthamoeba and muscle actin are quite similar, both contain 1 mole/mole of bound ADP, both polymerize rapidly in the presence of 2-5 mM $MgCl_2$ and slowly in the presence of 0.05-0.4M KCl to form filaments with the same reduced viscosity, both bind 1 myosin head per actin monomer, and both activate the Mg-ATPase of myosin but inhibit its K-ATPase. However, several significant differences have been

found. (1) Acanthamoeba actin but not muscle actin contains 1 residue of N^{ε}-methyl-lysines per actin monomer. (2) In 0.1M KCl (no Mg^{++}), polymerization of Acanthamoeba actin is much more temperature-dependent than that of muscle actin. At 5°C, the critical concentration for polymerization of Acanthamoeba actin is 0.4-0.5 mg/ml while that of muscle actin is only 0.1 mg/ml. (3) Acanthamoeba actin activates the Mg-ATPase of HMM (or S-1) only 1/3 as effectively as muscle actin. This is due to a difference in the amount of actin required for half-maximal activation. The extrapolated activation at infinite actin concentration is the same for both actins. These comparisons, and those in project Z01 J: 00412-01 LCB, suggest that Acanthamoeba and muscle actin, though similar, are functionally distinct molecules with significantly different properties.

Most recently we have begun to study the formation of heteropolymers of Acanthamoeba and muscle actin. We have found that sonicated muscle F-actin fragments can act as "seeds" for polymerization of Acanthamoeba actin and that Acanthamoeba F-actin fragments can fill the same role for muscle actin. The formation of heteropolymers has been confirmed by measuring the viscosity of mixtures of actin polymerized in 0.1M KCl at 5°C; it is significantly greater than the sum of the viscosities of the individual actins. Finally, the binding of tropomyosin to a 50-50 heteropolymer of Acanthamoeba and muscle actin in 5mM $MgCl_2$ differs quite markedly from a mixture of homopolymers. Studies of the interaction of tropomyosin and actin heteropolymers should be of particular interest because of the light they may shed on the cooperativity of the interaction of seven actin monomers with one molecule of tropomyosin.

Proposed Course of Research: Since we know some important properties of Acanthamoebaactin, we can now study the alteration of these properties by other proteins. The main focus for the coming year will probably be the interaction of Acanthamoeba actin, muscle actin, and copolymers of varying ratios with other proteins which may be involved in modulation of the actomyosin system. These include skeletal muscle tropomyosin and the troponins, the protein responsible for gelation of crude Acanthamoeba extracts at 30°C, high molecular weight "actin-binding proteins", spectrin, and tubulin. Possibly, one or more of these proteins or similar proteins may play an important role in translating the energy of the cytoplasmic actomyosin ATPase into cell motility.

Publications:

Gordon, D.J., Yang, Y.-Z., Eisenberg, E. and Korn, E.D.: Properties of actin isolated in high yield from Acanthamoeba castellanii. Cold Spring Harbor Symposium on Cell Motility, 1975. In press.

Gordon, D.J. and Korn, E.D.: Characterization of cytoplasmic actin isolated from Acanthamoeba castellanii by a new method. Journal of Biological Chemistry. In press.

Korn, E.D.: Workshop on membranes and their association with contractile proteins. In R. Goldmann, T.D. Pollard and J. Rosenbaum (eds) Cell Motility, Cold Spring Harbor Press, 1976, In press.

Korn, E.D.: Cytoplasmic actin and myosin. Trends in Biochemical Sciences 1: 55-58, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00504-11 LCB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Plasma Membrane and Phagosome Membrane of Acanthamoeba

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: E.D. Korn Chief, Lab. Cell Biol. LCB NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

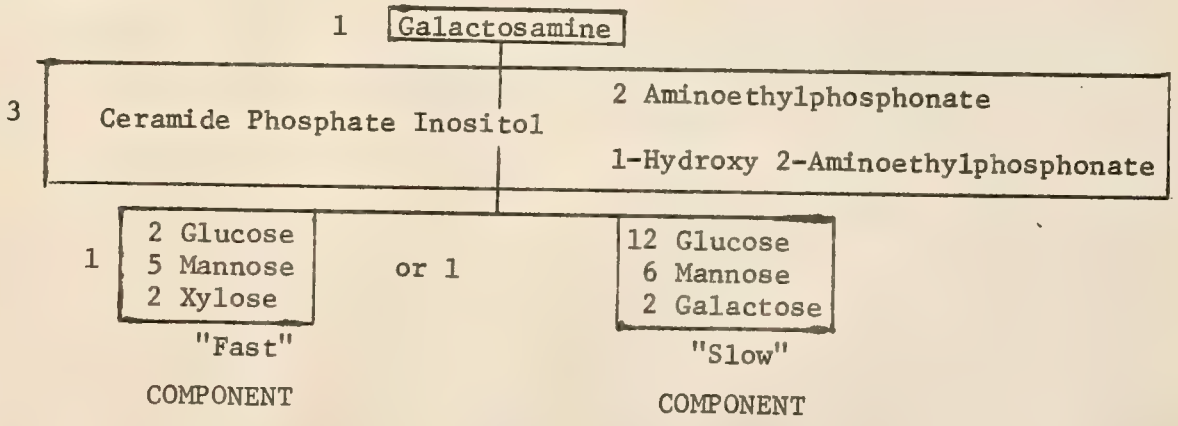
INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

SUMMARY OF WORK (200 words or less - underline keywords)

The structure of lipophosphonoglycan, a polymeric glycosphingolipid that makes up about 30% of the mass of the plasma membrane of Acanthamoeba castellanii has been studied. Working models for the structures of the two subunits that can be separated by dodecyl sulfate-polyacrylamide gel electrophoresis have been proposed as follows:



PHS-6040
(12-75)

Project Description:

Objectives: Ultimately, we hope to determine the complete chemical composition and macromolecular organization of the amoeba plasma membrane and related intracellular membranes and also to elucidate the mechanism of their fusions and interconversions. This project is related to the independent project of Dr. Blair Bowers (X01 HL 00502-11 LCB).

We know from previous work that the plasma membrane consists of approximately 25% phospholipid, 13% sterol, 35% protein and 29% lipophosphonoglycan. In previous years we have identified the phospholipids and sterols, characterized a number of plasma membrane enzymes concerned with phospholipid metabolism and demonstrated that lipophosphonoglycan is a novel macromolecule probably best considered as a polymeric glycosphingolipid.

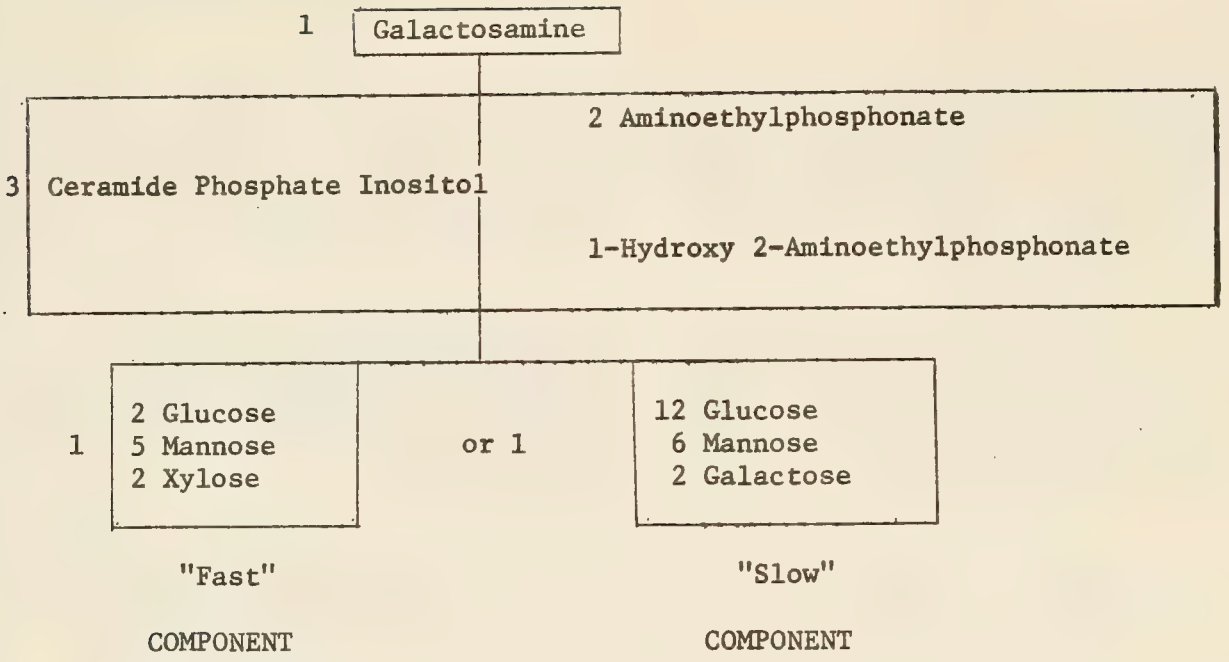
Lipophosphonoglycan contains 26% neutral sugars (glucose, mannose, galatose, xylose;5:4;1:1) 3.3% aminosugars (galactosamine, glucosamine; 1:1) 10% aminophosphonic acids (2-aminoethylphosphonate, 1-hydroxy-2-aminophosphonate) 3.2% acid - hydrolyzable phosphate and 14% long chain fatty acids (33% saturated and unsaturated, normal and branched C_{16}-C_{28} and 67% normal and branched saturated 2-hydroxy C_{22}-C_{28}) 8% inositol and 13% C_{24} and C_{25} phytosphingosines accounting altogether for about 80% of the mass of the molecule.

From previous work, we know that lipophosphonoglycan can be separated by dodecylsulfate polyacrylamide electrophorosis into two components. This year we have analyzed the separated subunits and can propose tentative general structures for each of them.

Methods and Findings:

Purified lipophosphonoglycan was separated into two components by preparative dodecyl sulfate-polyacrylamide slab electrophoresis. The two fractions obtained were analyzed for total sugars by the phenol-sulfuric reaction, for individual neutral sugars by gas-liquid chromatography of their reduced, acetylated derivatives, for inositol by inositol dehydrogenase, for fatty acids and phytosphingosines by thin-layer and gas-liquid chromatography, for total and acid-hydrolyzable phosphate, and for glycerol by glycerol dehydrogenase and gas-liquid chromatography.

Previous identification of glycerol as a component of lipophosphonoglycan was not confirmed. Based on the stoichiometry of the analyses and previous data, the following working structure for the two subunits was reached ("fast" and "slow" refer to their electrophoretic mobility).



It is proposed that both subunits consist of a trimeric unit containing ceramidephosphateinositol to which the aminophosphonates are linked, possibly through the inositol. The linkages holding this trimer together are not known. To this backbone would be linked one galactosamine and an oligosaccharide. The oligosaccharide moiety of the "fast" component would contain glucose, mannose and xylose in the ratio of 2:5:2 and the "slow" component oligosaccharide would contain glucose, mannose and galactose in the molar ratio of 12:6:2. Glucosamine has not been accounted for in the hydrolysis products and, therefore, is ommitted from these tentative structures. These working models satisfy the qualitative and quantitative analytical data.

Proposed Course of Research: More time will be spent on this project next year. It is hoped to separate the two components in large enough yield to allow the structural analysis to proceed.

Publications:

Batzri, S., and Korn, E. D.: Interaction of phospholipid vesicles with cells: Endocytosis and fusion as alternate mechanisms for the uptake of lipid-soluble and water-soluble molecules. J. Cell. Biol. 66: 621-634, 1973.

Victoria, E. J. and Korn, E. D.: Plasma membranes and soluble lysophospholipaes of Acanthamoena castellanii. Arch, Biochem. Biophys. 171 255-258, 1975.

Dearborn, D. G., Smith, S. and Korn, E. D.: Lipophosphonoglycan of the plasma membrane of Acanthamoeba castellanii: inositol and phytosphingosine composition and general structural characteristics. J. Biol. Chem. In press.

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Editor: Korn, E. D. (Ed.): Methods in Membrane Biology, New York, Plenum Press, 1975, Vol. 4, 298 pp.

Editor: Korn, E. D. (Ed.): Methods in Membrane Biology, New York, Plenum Press, 1975, Vol. 5, 199 pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00506-01 LCB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Temperature-dependent Gelation and Retraction of Acanthamoeba Extract

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Maruta	Visiting Fellow	LCB NHLI
	E.D. Korn	Chief, Lab. Cell Biol.	LCB NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.45	1.45	0

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied a temperature-dependent gelation and retraction of Acanthamoeba extract in order to understand the mechanism of cell motility. We isolated several new proteins, four gelation factors and one retraction factor, from Acanthamoeba by procedures including DEAE-cellulose chromatography, ammonium sulfate fractionation, gel-filtration of Bio-Gel 1A 1.5m and hydroxylapatite chromatography. The former factors catalyze the gelation of actin and the latter induces the retraction of the actin-gel in the presence of ATP and Mg++. Our work will focus on the understanding of the understanding of the nature of interaction of these proteins with actin and the roles in motile functions of non-muscle cells.

PHS-6040
(12-75)

Project Description:

Objectives: In the years 1968-1975, studies in this laboratory on Acanthamoeba castellanii provided some of the earliest and best evidence for the presence of "contractile" proteins (actin, myosin and cofactor protein) in the cytoplasm of non-muscle cells. Acanthamoeba myosin is unique among known myosins in having a much lower molecular weight (about 180,000 vs 450,000) and in requiring a cofactor protein for actin-activation of its Mg-ATPase. (A similar cofactor protein has more recently been found in pulmonary macrophage.) Acanthamoeba actin is by far the best characterized cytoplasmic actin and shows interesting differences from muscle actin with which it is so generally similar (see projects ##Z01 HL 00501-02 LCB and Z01 HL 00412-01 LCB). Previous work in this laboratory has shown that Acanthamoeba actin is strongly associated with the cytoplasmic surface of the plasma membrane and this observation has been confirmed in many other cells. One possible mechanism for actin-myosin based motility systems would be for the plasma membrane to function in some way analogously to the Z-line of muscle together with sliding thin (actin) and thick (myosin) filaments. Although this model may be applicable in some cytoplasma systems there are difficulties in generalizing it to all cell motile systems. For example, some cells seem to have actin but no myosin, in all cells there is much less myosin than actin (they are equimolar in muscle), myosin thick filaments have rarely if ever been detected and microfilaments (actin), although abundant, are usually not oriented as in muscle. On the other hand, the cytoplasmic systems may have a number of additional associated proteins that are not present in muscle; the role of these proteins is not clear.

Recently, other investigators have observed that cold extracts of several cells, including Acanthamoeba, slowly form solid gels (or more rapidly if warmed) which will retract upon warming. This in vitro gelation and retraction may form an appropriate model for cytoplasmic motile events that could well involve the "gelation" and "retraction" of cytoplasmic microfilaments in the ectoplasm underlying the plasma membrane. It has been supposed by others that a recently-described high molecular weight protein, "actin-binding protein," and myosin may be involved in these gelation and retraction events but a systematic, quantitative study of the active components has not yet been done. We have now initiated such an investigation with Acanthamoeba.

Methods Employed and Preliminary Findings:

Gelation factors are semi-quantitatively assayed by their ability to (1) cause solutions of muscle F-actin to gel (2) increase the viscosity of muscle F-actin and (3) inhibit the activation of HMM-ATPase by muscle F-actin. Retraction factors are qualitatively assayed by their ability to cause retraction of a gel formed by a mixture of muscle F-actin and an appropriate gelation factor. Using these assays we have been able to isolate several new proteins, four highly purified gelation factors and one partially purified retraction factor, from extracts of Acanthamoeba using techniques of DEAE-cellulose chromatrography, ammonium sulfate fractionation, gel filtration and chroma-

tography on hydroylapatite. The gelation factors have approximate molecular weights of 78,000, 64,000, 54,000 and 23,000. The first three may be dimers. The retraction factor may be a 180,000 dalton polypeptide detected on dodecyl sulfate-polyacrylamide electrophoresis of active fractions.

The gelation of muscle F-actin induced by each of the gelation factors is maximal at 30°C which, perhaps coincidentally, is the optimal temperature for growth of the amoeba. The molar ratio of gelation factor to actin required to form a solid gel is about 1:1000. No formation of covalent bonds between actin molecules or between actin and gelation factor has been detected. The gelation factors each inhibit actin-activated HMM-ATPase but not HMM-ATPase, alone. At lower concentrations of actin than required to form a gel, an increase in viscosity is induced by the gelation factors. Contrary to suggestions of others based on more indirect reasoning, we can find no evidence for a role of a high molecular weight "actin-binding protein" in the gelation although the Acanthamoeba extracts contain such a protein and it is associated with the pellet of retracted, gelled extracts.

Preliminary evidence suggests that the retraction factor is not the Acanthamoeba myosin described previously by this laboratory since it has a higher molecular weight (180,000 vs 140,000 for the myosin heavy chain), has only very low, if any, Ca-ATPase activity and no K-EDTA ATPase activity, and can be separated from Acanthamoeba myosin by chromatography on DEAE-cellulose, ATP-agarose and Biogel A 1.5m. The retraction factor does bind strongly to the retracted actin.

Proposed Course of Research: We will continue to purify the gelation and retraction factors until purity is reached and will study their interaction with actin and myosin in relation to the processes of cell motility.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00404-17 LCB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Proteolytic fragmentation of fibrinogen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Elemer Mihalyi, Staff Investigator, LCB, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

SUMMARY OF WORK (200 words or less - underline keywords)

Molecular weights and associating properties of fragments D and E of fibrinogen. The work started last year on the characterization of isolated D and E fragments will be continued. Molecular weights of these fragments obtained from bovine or human fibrinogen, with either trypsin or plasmin, will be determined by sedimentation equilibrium. It was found in these experiments that homologous fragments show appreciable association. The associating properties also will be investigated using the sedimentation equilibrium method.

PHS-6040
(12-75)

Project Description:
Objectives:
Intermolecular interactions leading to formation of fibrin gels are localized in the D and E regions of the fibrin molecule (Kudryk et al. J. Biol. Chem. 249, 3322, 1974). The present investigations were undertaken to establish whether or not these regions interact also in solution, when isolated in fragments derived by proteolytic digestion of the fibrinogen molecule.

Methods Employed:
Proteolytic digestion, fractionation on Sephadex G200 columns, sodium dodecyl sulfate -- polyacrylamide gel electrophoresis with UV-scanning, computer resolution of the elution curves.

Major Findings:
Fibrinogen digests, at the completion of cleavage into the D and E fragments, were fractionated on a Sephadex G-200 column. Fragments D and E were estimated specifically across the main peak either by selective heat coagulation of fragment D or by SDS-polyacrylamide gel electrophoresis. The resulting elution pattern of fragment E in the presence of fragment D was compared to that of isolated fragment E. With tryptic digests of bovine fibrinogen the patterns were identical, indicating no interaction of the two fragments. With plasmin digests of human fibrinogen, on the contrary, fragment E in the mixture eluted earlier, in an asymmetric boundary, extending across the whole main peak. This finding suggests some interaction between the two fragments. With tryptic digests of bovine fibrinogen fragment D can be obtained in a pure state by pooling the first half of the main peak of the Sephadex G-200 fractionation. The second half of the peak, after heat coagulation of fragment D, yields fragment E in a pure state. This method cannot be applied to plasmic digests of human fibrinogen because of the spreading of fragment E through the entire D peak. However, fragment E can be obtained in a pure state by heat coagulation of this peak.

Project:
This phase of the project completed. Other aspects of the proteolytic fragmentation of fibrinogen will be continued.

Publications:
Mihalyi, E. and Towne, D.W.: Behavior of fibrinogen fragments D and E in gel filtration experiments. Thromb. Res. 8:1-15, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00405-02LCB

PERIOD COVERED
July 1, 1975 through June 20, 1976

TITLE OF PROJECT (80 characters or less)
Circular Dichroic Studies on Denatured Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: F.H. White, Jr., Staff Investigator, LCB, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology, NHLI

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

It has long been established that development of conformational structure is dependent on the amino acid sequence, although the exact relationship (which would make possible the precise prediction of native conformation from primary structure, as well as the mechanism by which it forms) still eludes us. It has long been believed that native conformation develops directly from the randomly coiled chain of the denatured protein. Recent observations in several laboratories indicate, however, that denatured chicken egg white lysozyme possesses secondary structure whith resembles that of the native protein, although much of the native structure has been destroyed. The present project is an attempt to characterize the structure of reduced lysozyme by its circular dichroic behavior. It has been found that two to three times more β-structure exists in the reduced chain than is present in the native protein, although α-helix is much diminished. The significance of this observation lies largely in the possibility that the reduced structure may function as a "precursor conformation" that would influence the resumption of native structure when the reductive process is reversed (or after synthesis of the reduced chain in the living cell). This investigation will be extended to other proteins.

PHS-6040
(12-75)

Project Description:

Objectives: To characterize the conformational structure of a fully reduced alkylated protein.

Methods:

1. Procedure for reduction of disulfide bonds in protein is similar to that already published (F.H. White, Methods in Enzymol. 25, 387 (1972)).

2. Procedures for alkylation of the SH groups (with iodoacetic acid (IAc) or iodoacetamide (IAm)) that result from reduction are also published above. In addition the method of J. Swan and S. Wright (Aus. J. Chem. 24, 777 (1971)) was used for the alkylation of SH groups with triphenylvinylphosphonium bromide (TVP).

3. The processing of data from circular dichroic studies was accomplished with a PDP-10 computer employing the "MLAB" curve fitting system. Reference data for this study were taken from four sources:

a) N. Greenfield and G. Fasman, Biochem. 8, 4108 (1969).
b) I.P. Saxena and D.B. Wetlaufer, Proc.Nat.Acad.Sci. 68, 969 (1971).
c) Y.-H.Chen, J.T. Yang, and H. Martinez, Biochem. 11, 4120 (1972).
d) Y.-H. Chen, J.T. Yang, and K.H. Chau, Biochem. 13, 3350 (1974).

Major Findings:

1. Observations have been made on the reduction of proteins. Reduction under conditions previously thought to render a protein into the reduced state (i.e., all disulfide bonds broken) instead result in approximately 95% reduction. Several other workers have studied the circular dichroic behavior of various proteins in the reduced state, assuming them to be fully reduced. The present study has shown that the 95% level is not satisfactory for this purpose. The present solution to the problem involves a further reduction (after the initial reductive step) followed by exhaustive alkylation with IAm, IAc, or TVP. By all analytical methods employed, reduction was then complete.

2. Reference curves for the circular dichroic behavior of pure α-helix, pure β-structure and pure unordered structure are essential for computer analysis. Several sets of published curves have been tried, with the conclusion that those of Greenfield and Fasman (Biochem. 8, 4108 (1969)) are the best suited for computer analysis of circular dichoic behavior of native and reduced lysozymes. The picture is less clear for other proteins studied.

3. Evidence for remaining secondary structure has been found for every reduced protein thus far studied, whereas formerly it was widely believed that cleavage of disulfide bonds (or other denaturation) would render a protein chain into the randomly coiled form.

4. For lysozyme, secondary structure in the reduced state was characterized by 2 to 3 times more β-structure than exists in the native state. The contents of both α-helix and β-structure increased with increasing pH from 2 to the limit of solubility of each reduced derivative.

5. The content of β-structure in reduced lysozyme derivatives was greatly enhanced by methanol (62% of the chain length as β-structure in 50% methanol.

6. The other proteins studies were ribonuclease, papain, elastase, nuclease from Staphyloccus aureau, chymotrypsin, and cytochrome c. Some of these appear to follow the pattern of lysozyme on reduction, insofar as there is either a preservation or buildup of β-structure, while others lose both α-helix and β-structure, although never entirely. Typical of the latter group is ribonuclease.

7. Contrary to previous experience (Annula Report for Project No. Z01HL 00405-01LCB, 1975) the present computer analysis indicates that 8M urea or 6M guanidine destroys α-helix in reduced lysozyme but does little to the β-structure. The denaturing effect is enhanced by lowering the temperature to 5°.

Significance:

It has long been believed that reduction of disulfide bonds would result in the formation of a random coil. The present results suggest that such a form rarely if ever occurs.

The presently observed surfeit of β-structure in the reduced state indicates a conformation characteristic of that state and that this conformation is not merely residual in nature (i.e., a partially destroyed native conformation). The function, if any, of this newly found structure remains to be clarified. Its significance lies largely in the possibility that it may function as a 'precursor conformation' that would influence the resumption of native structure when the reductive process is reversed (or after the reduced protein chain has been newly synthesized in the living cell).

It has long been established that development of conformational structure is dependent on the amino acid sequence, but the exact relationship still eludes us. The present results suggest a new approach to this problem.

If, in actuality, the conformation shown to be characteristic of the reduced state of lysozyme functions as a precursor for the formation of native structure, a study of this function may lead to a better understanding of the mechanism by which the native coiling and folding of the protein chain develop.

Ultimately it should be possible to predict the conformation of a native protein molecule, having only a knowledge of its amino acid sequence.

Proposed Course:

The computer study of structure in reduced proteins other than lysozyme will be continued, with efforts made to develop reference data that more accurately describe the structural moieties present after reduction.

Degradative procedures will be employed on reduced alkylated proteins, followed by isolation and circular dichroic studies of the resulting peptides. Thus the amino acid sequences involved in the β-structure characteristic of the reduced state may be clarified.

Publications:

White, F.H., Jr.: Preferential tritium labelling of binding site residues in alpha chymotrypsin by exposure of the 1,3-^{3}H-diisopropylphosphoryl derivatives to tritiated hydrogen sulfide. J. of Labelled Compounds 11: 11 (1975).

White, F.H., Jr.: Studies on secondary structure in chicken egg white lysozyme after reductive cleavage of disulfide bonds. Biochemistry: in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00505-10 LCB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cytology of Acanthamoeba

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Bowers	Research Biologist	LCB NHLI
OTHER:	A. Ryter	Visiting Scientist	LCB NHLI
	A.H. Chagla	Visiting Fellow	LCB NHLI

COOPERATING UNITS (if any)

LB NHLI (Project Z01 HL 00205-21 LB)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

NHLI, NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.8	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

Our long range goals are to study the interrelationships and possible interconversions of internal membrane systems with those of the plasma membrane during endocytosis in the soil ameba, Acanthamoeba castellani. We are using primarily a morphological approach that includes electron microscopy, freeze-fracture replication and cytochemistry. The present report describes the cytochemical localization of acid phosphatase activity which makes it possible to study the timing of lysosome-phagosome fusions. In a collaborative study, the fine structure of the bacterium Methanococcus vannielii was described with emphasis on the cell wall structure.

PHS-6040
(12-75)

Project Description:

Objectives: To elucidate the structural basis of biochemical and physiological events in the soil ameba, Acanthamoeba castellani. Our current emphasis is on understanding more about the interrelationships and possible interconversions of internal membrane systems with those of the plasma membrane through biochemical and morphological studies of endocytosis and through morphological studies of the membranes with freeze-fracture replication and enzyme cytochemistry.

Methods Employed:

1. Transmission electron microscopy is being used for the study of fixed and embedded cells and for evaluation of isolated cell fractions.

2. The light microscope with phase contrast and Nomarski interference optics and time-lapse cinematography are being used for study of living cells.

3. The technique of freeze-fracture replication (also dependent on transmission electron microscopy) is being used for the study of surface and internal membrane morphology.

4. Routine and special biochemical and cytochemical procedures are carried out as necessary to prepare specimens for observations with the microscopic procedures and to supplement morphological data.

5. Stereological procedures are being used to quantitate and interpret electron micrographs.

Major Findings:

1. In seeking an ultrastructural "marker" for one of the internal membrane systems, we attempted to localize one of the lysosomal hydrolases by ultrastructural cytochemical procedures. Dr. A. Ryter, guest worker, performed this study. The technical difficulties of this procedure are considerable. In the course of overcoming some of the problems inherent in the method, it was ascertained that one of the major difficulties was impermeability of the Acanthamoeba plasma membrane to the cytochemical substrate. The cell density of the cultures was found to have a marked effect on the ease of cytochemical demonstration of acid phosphatase. In light microscopic assays, cells from cultures of low cell density (less than 4×10^5 cells/ml) gave almost no reaction unless treated to increase membrane permeability, whereas cells from cultures of high density gave a high percentage of reactive cells. By using cultures of high cell density it was possible to obtain what appears to be reliable localizations of acid phosphatase activity at the electron microscopic level. Although there is ample biochemical evidence for the existence of lyosomes in Acanthamoeba, this is the first localization at the EM level of a lysosomal enzyme in the trophozoite of Acanthamoeba. Acid phosphatase activity was localized in

three types of structures: small vesicles of the Golgi apparatus, large cytoplasmic vacuoles ("digestive" vacuoles) and in cytoplasmic structures which are unidentified as to function. These latter structures are the same size as mitochondria, are bounded by a single membrane and contain small tubules. They are very similar in appearance to structures that have been identified as peroxisomes in a closely related ameba, Hartmanella culbertsonii (Childs, J. Histochem. Cytochem. 21:26.1973). If they represent a similar organelle in Acanthamoeba, the presence of phosphatase activity in them is puzzling. The function of these structures in Acanthamoeba requires further study.

With the successful cytochemical localization of an enzyme marker for lyosomes, the timing of the phagosome-lysosome fusions could be investigated. Acanthamoeba cells were fixed and reacted cytochemically at intervals after ingestion of yeast. The relative numbers of acid-phosphatase positive and negative vacuoles were determined from counts in the light microscope on whole cells and from analysis of electron micrographs. Positive phagosomes (i.e. phagolysosomes) increased rapidly during the initial hour of uptake. After 90 min., when uptake was essentially complete, the number of phosphatase-positive phagolysosomes amounted to roughly half the phagosome population. This proportion remained relatively constant for the next four hours. The number of acid-phosphatase-positive vacuoles in the cytoplasm decreased with phagocytosis, but the kinetics of this disappearance do not suggest a fusion of vacuoles with phagosomes. A portion of the phagosomes became phosphatase-negative as digestion of their content proceeded. Whether the negative reaction was due to an alteration of membrane permeability or to a true loss of enzyme activity could not be determined from the present data. Biochemical tests for acid phosphatase activity in the medium were negative, suggesting that few if any active enzymes were released with defecation of indigestible residues. There was also no evidence from the chemical assays for increase of acid phosphatase activity in cells loaded with particles.

2. In collaboration with T. C. Stadtman and J. B. Jones, (LB NHLI) we have investigated the morphology of an unusual bacterium, Methanococcus vanniellii. Methanococcus is a motile, methane-producing organism that is a strict anerobe. it grows in media of low inoic strength and is resistant to lysis by media of low osmolarity, but it is fragile and easily ruptured by mechanical stresses. Jones and Stadtman have shown that Methanococcus is not sensitive to antibiotics that interfere with the synthesis of the peptidoglycan layer in other bacteria and does not incorporate diaminopimelic acid, suggesting that this organism lacks a peptidoglycan layer. It was of interest, therefore, to examine the ultrastructure of Methanococcus to determine if it has any wall structure, or if it is analogous to stable L-forms, which appear to lack cell walls.

The organism was examined in thin sections, by freeze-fracture replication, by negative staining and in shadowed preparations.

Shadowed whole cells showed that Methanococcus has one or more polar tufts of flagellae. Each tuft may contain up to six flagellae. In thin sections it was observed that short regions of the plasma membrane and the subjacent cytoplasm show a differentiated, regular substructure. By analogy with other bacteria, these may be the areas that give rise to the flagella tufts.

In fixed and embedded material, cells grown on agar were of irregular shape and contained electron dense inclusions similar in appearance to animal glycogen. Cells in suspension culture were also examined and differed from agar-colony cells in that they were more nearly spherical in shape and lacked the dense inclusions. In both types of preparation, the plasma membrane clearly had a layer of material external to it. This layer is approximately 18 nm thick and stains more strongly on the outer edge. It was most clearly seen in those cells that were poorly fixed. In well-fixed cells it appeared as a dense line in close apposition to the plasma membrane. The trilaminer structure of the plasma membrane is difficult to resolve in this organism.

The structure of the cell wall material was best revealed in freeze-fracture replicas. Two fracture planes occurred, one along the outside of the wall and a second that presumably passed through the hydrophobic region of the plasma membrane. The cytoplasmic face of the plasma membrane contained a dense array of intramembranous particles (IMP). Scattered about on the fracture face are short sets of ridges. Various patterns of these special areas can be seen. In one case three stacks delimited a triangular area of the membrane. The opposite (extracellular) fracture face appeared smooth, except for sets of grooves that appear to match the ridges on the cytoplasmic fracture face.

The outer surface of the wall showed linear arrays of subunits in a tetragonal pattern. The subunits have a center to center spacing of 10.8 nm. The subunit composition of the extracellular coat in this bacterium is similar to that seen, so far, in about a dozen other bacteria. There is some evidence that the subunits are composed mainly of protein. This distinctively patterned wall material lies outside the peptidoglycan layer in gram positive bacteria and outside the outer wall in gram negative bacteria. A similar pattern has also been seen on another highly specialized bacterium, Halobacterium halobium, in which there is good evidence that a peptidoglycan component in the wall is lacking.

Significance to Bio-medical Research: Phagocytosis is a major mechanism of defense against infection and pinocytosis promises a means of therapy in certain storage diseases in which lysosomal enzymes are defective. Both of these processes take place at high rates in the ameba and can easily be studied in Acanthamoeba.

Changes in cell surface properties appear to play a major role in proliferation of cancerous cells. Acanthamoeba is in some senses an analog of transformed cells and thus information on the organization of its surface membrane may prove useful in understanding malignant cells.

Proposed Course of Research: The interrelationships of surface membrane and internal membrane systems in Acanthamoeba castellanii will be studied by cytochemical and stereological techniques and with freeze-fracture replication.

Publications:

Duran, A., Bowers, B. and Cabib, E.: Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc. Nat. Acad. Sci. 72: 3952-3955, 1975.

Cabib, E. and Bowers, B.: Timing and function of chitin synthesis in yeast. J. Bacteriol. 124: 1586-1593, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00407-03LCB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies on the interaction of actin with spin-labeled myosin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Sally A. Mulhern, Staff Fellow, LCB, NHLI
Other: Evan Eisenberg, Head, CP, LCB, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1	.25

SUMMARY OF WORK (200 words or less - underline keywords)

Myosin was modified at the SH_1 group with piperidinyl nitroxide, the spin label analogue of iodoacetamide. The proteolytic digestion product of spin-labeled myosin, spin-labeled HMM showed only three-fold actin activation of its Mg-ATPase by actin in contrast to 100-fold activation for unmodified HMM. Furthermore ultracentrifuge experiments demonstrated that when the actin activated ATPase was close to V_{max} most of the spin-labeled HMM was not bound to actin. These results were also obtained for HMM labeled at the SH_1 with N-ethylmaleimide indicating that the effect of blocking the SH_1 group of myosin does not seem to depend significantly on the nature of the group used for the blocking.

PHS-6040
(12-75)

Project Description:

Objectives:

The interaction of myosin with actin and ATP is the central reaction involved in muscle contraction. This reaction has been studied with myosin labeled at the SH_1 group with piperidinyl nitroxide, the spin label analogue of iodoacetamide (Seidel and Gergely; Cold Spring Harbor Symposium, 1972, Vol. 37. Studies have also been done on spin-labeled myosin employing saturation transfer electron paramagnetic resonance to determine the motion of subfragment-1 in myosin and its supramolecular complex. Since blocking the SH_1 group of myosin with N-ethylmaleimide greatly affects its APTase activity, it is important to determine how blocking the SH_1 group with the spin label affects the ATPase activity of myosin in order to interpret the spin-labeled myosin data. In the present study we compared heavy meromyosin blocked with NEM (NEM-HMM) and HMM blocked with the spin label. We previously have performed detailed studies on the ATPase of NEM-HMM both with and without actin present and also studied the binding of NEM-HMM to actin in the presence of ATP. We found that NEM-HMM is only 3-fold activated by actin in comparison to 100-fold for normal HMM. We also found that very little NEM-HMM bound to actin when the ATPase is close to V_{max}. On this basis we postulated that NEM-HMM like unmodified HMM exists in a refractory state which cannot bind to actin during ATP hydrolysis. However we postualted that the rate of conversion from the refractory to the nonrefractory state is lower for NEM-HMM than for normal HMM. In the present study we were interested in determining whether HMM labeled with the spin label showed the same behavior as NEM-HMM in its interaction with actin.

Methods Employed and Major Findings:

Myosin was labeled with increasing amounts of spin label under conditions where the spin label reacts with sulfhydryl groups. Each sample was assayed for Ca^{++} and EDTA-ATPase activity. The Ca^{++} ATPase activity increased 8-fold at a spin lable to myosin ratio of 10:1 and remained constant at higher ratios of spin label to myosin whereas the EDTA-ATPase activity was 90% inhibited at a 10:1 ratio. This suggests that the spin label has reacted at the SH_1 group of myosin since similar ATPase activities in the presence of Ca^{++} and EDTA were observed for myosin modified at the SH_1 group with NEM. The only difference was that in general the ATPase activity of the spin-labeled HMM was about twice that of the NEM-HMM ATPase activity. However like the NEM-HMM the spin-labeled HMM showed only three-fold actin-activation of its Mg-ATPase by actin in contrast to 100-fold activation for unmodified HMM. Furthermore ultracentrifuge experiments demonstrated

that as for NEM-HMM, when the actin activated ATPase was close to V_{max} most of the spin-labeled HMM was not bound to actin. Thus the effect of blocking the SH_1 group of myosin does not seem to depend significantly on the nature of the group which is used for the blocking.

Proposed Course of Research:

Since many fluorescence depolarization studies are being done on myosin by utilizing a myosin which has been labeled at the SH_1 group with a fluorescence dye it would be of interest to determine if this dye-modified myosin also has the same properties as NEM-HMM. This will be ascertained by determining the Ca^{++} and EDTA-ATPase activities of the labeled myosin and also studying its actin-activated ATPase activity and its binding to actin in the presence of ATP.

Publications:

Mulhern, S., Eisenberg, E., and Kielley, W.W. Interaction of actin with N-ethylmaleimide modified heavy meromyosin in the presence and absence of adenosine triphosphate. Biochemistry 14:3863 (1975).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00409-06LCB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
The interaction of actin and myosin.

NAMES, LABCRATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Evan Eisenberg, Head, CP, LCB, NHLI

Other (theoretical) Terrell Hill, NIAMD
(Stop flow studies) S. Chock, Staff Fellow, LCB, NHLI
P.B. Chock, Staff Investigator, LB, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1	0.25

SUMMARY OF WORK (200 words or less - underline keywords)

Our major effort over the next year will be to determine the relationship of the initial P_i burst to the transition from the refractory to the non-refractory state. The P_i burst has been studied most thoroughly at 20° and it appears that at this temperature the P_i burst is considerably faster than the steady-state ATPase rate. To make certain of this we will study both the P_i burst and the rate of the transition from the refractory to the non-refractory state in a single cycle of ATP hydrolysis by acto S-1 at 20°. To study the P_i-burst we will use a 3-syringe stopped-flow apparatus. With this technique we should also be able to determine if the S-1 is homogeneous, i.e. whether all of it shows the initial P_i burst and follows the same kinetics or whether as postulated by Tonomura, two kinds of S-1 occur. In these experiments the S-1 will be purified suing an ATP-affinity column to rule out the presence of denatured S-1. We also plan to continue our collaboration with Terrell Hill, NIAMD, quantitating our cross-bridge model to determine whether it will fit with current physiologic data.

PHS-6040
(12-75)

Project Description:

Objectives:

It is now generally recognized that contraction of muscle involves the interaction of the two proteins actin and myosin with ATP. It is therefore of considerable importance to determine the nature of the interaction which occurs between actin and myosin in vitro as ATP is hydrolyzed. In particular it is of importance to elucidate the steps occurring during the hydrolytic cycle, since these steps may correspond to the steps of the contractile cycle in vivo. This is difficult to accomplish with myosin because it occurs as insoluble filaments at low ionic strength. However, heavy meromyosin (HMM) and subfragment-1 (S-1), double and single headed proteolytic digestion of myosin respectively, are soluble at low ionic strangth and therefore their interaction with actin can be more easily studied. In the present study we continued our investigation of the refractory state of HMM and S-1, a state which we discovered occurs during the cycle of interaction of myosin with actin and ATP during which the myosin head is unable to bind to actin. Only when the refractory state transforms to the non-refractory state is the myosin able to interact with actin and the transition from the refractory state to the non-refractory state seems to be one of the major rate limiting steps in the cyclic interaction of myosin with actin and ATP. The occurrence of a refractory state has considerable implications for the actin-myosin cycle which occurs in vivo and therefore in the present study we devoted considerable effort to proving its existence and determining its chemical nature using stopped-flow techniques. In addition, in collaboration with Terrell Hill, NIAMD, we have developed a complete model of cross-bridge actins which shows the significance of the refractory state for muscle contraction in vivo.

Methods Employed and Major Findings:

The original finding which led us to postulate the existence of a refractory state is that considerable dissociation of the acto-HMM or acto-S-1 occurs even under conditions where the actin maximally activates the HMM or S-1 ATPase as shown by ultracentrifuge and ATPase studies. As described in the project report Z01HL 00410-03LCB stopped-flow data has confirmed the existence of the refractory state. This data has suggested that the acto-1 complex dissociates and reassociates each time an ATP molecule is hydrolyzed and furthermore the data has shown that at high actin concentration the transition from the refractory to the non-refractory state is rate limiting

Thus the reassociation of the S-1 and actin is quite slow while the dissociation of the acto S-1 complex by ATP is very rapid in vitro.

In 1957, A. Huxley presented a cross-bridge model of muscle contraction in which the rate of association of the myosin cross-bridge with actin was postulated to be quite slow compared to the rate of dissociation of the cross-bridge following its work stroke. Keeping the rate of attachment slow was important since it limited the rate of ATP hydrolysis when the muslce contracted at relatively high velocity. Thus the efficiency of muscle contraction is kept relatively high over a broad range of velocity.

In the cross-bridge model of muscle contraction we have developed with Terrell Hill, the biochemical cycle we determined in vitro is used as a basis for a complete model of cross-bridge actin. In this model, the low rate of the transition from the refractory to the non-refractory state is used to limit the rate of attachment of the cross-bridge and thus produces a high efficiency contraction at relatively high shortening speed. Two other features of this model are that rather than placing an independent elastic element in the cross-bridge as suggested by Huxley and Simmons, the cross-bridge is postulated to "rock" about either of two stable angles, depending on the chemical state of the cross-bridge. In addition, the model suggests that all of the free energy of ATP hydrolysis is "stored" in the dissociation of the actin and myosin by ATP with the work step being driven by a restoration of a strong actomyosin link as the cross-bridge rocks over toward a stable 45° angle. Over the last several months we have worked out a complete description of this model and it will shortly be submitted for publication.

Proposed Course of Research:
Our major effort over the next year will be to determine the relationship of the initial P_i burst to the transition from the refractory to the non-refractory state. The P_i-burst has been studied most thoroughly at 20° and it appears that at this temperature the P_i burst is considerably faster than the steady-state ATPase rate. To make certain of this we will study both the P_i burst and the rate of the transition from the refractory to the non-refractory state in a single cycle of ATP hydrolysis by acto S-1 at 20°. To study the P_i-burst we will use a 3-syringe stopped-flow apparatus. With this technique we should also be able to determine if the S-1 is homogeneous, i.e. whether all of it shows the initial P_i burst and follows the same kinetics or whether as postulated by Tonomura, two kinds of S-1 occur. In these experiments the S-1 will be purified using an ATP-affinity column to rule out the presence of denatured S-1. We also plan to continue our collaboration with Terrell Hill, NIAMD, quantitating our cross-bridge model to determine whether it will fit with current physiologic data.

Significance to Bio-medical Research:
This work is aimed at gaining a better understanding of the basic mechanism of muscle motility and the control of this motility, phenomena which occur not only in skeletal muscle, but also in such diverse systems as cardiac muscle, arterial smooth muscle, platelets and perhaps within all cells where protoplasmic streaming occurs.

Publications:

Fraser, A.B., Eisenberg, E., Kielley, W.W., and Carlson, F. D. The interaction of heavy meromyosin and subfragment 1 with actin. Physical measurements in the presence and absence of adenosine triphosphate. Biochemistry 14:10, 2207 (1975).

Eaton, B.L., Kominz, D.R. and Eisenberg, E. Correlation between the inhibition of the acto-heavy meromyosin ATPase and the binding of tropomyosin to F-actin: Effects of Mg^{2+}, KCl, Troponin I, and Troponin C. Biochemistry 14:12, 2718 (1975).

Hill, T.L. and Eisenberg, E. Reaction free energy surfaces in myosin-actin-ATP systems. Biochemistry (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01HL 00410-03LCB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mechanism of myosin and actomyosin-Mg-ATPase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Stephen P. Chock, Staff Fellow, CP, LCB, NHLI

Other Evan Eisenberg, Head, CP, LCB, NHLI
Sally Mulhern, Staff Fellow, CP, LCB, NHLI
P.B. Chock, Staff Investigator, LB, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1	0.25

SUMMARY OF WORK (200 words or less - underline keywords)

Our studies will focus on understanding the nature of the actin-S-1 interaction in the presence and absence of ATP and the nature of the steps that occur in the actomyosin ATPase cycle. Efforts will also be made to understand the effect of the SH_1 group on the catalytic nature of myosin Mg^{++}-ATPase. The use of computer curve fitting in understanding the kinetic nature of different mechanistic models will be explored.

PHS-6040
(12-75)

Project Description and Objectives:

Recent progress in our understanding of the mechanism of muscle contraction has been significantly advanced by the use of presteady state and steady state stopflow techniques in the kinetic studies of both myosin and actomyosin Mg-ATPase in vitro. The use of the presteady state approach especially, has led to the discovery of various kinetic intermediates through which enzymes must go during each cycle of ATP hydrolysis. As a result of this, various mechanistic models, including our own, have been proposed. However, there are still many disagreements among the various models and many experimental observations left unexplained. Our ultimate objective is, therefore, to develop a complete kinetic model for myosin and actomyosin Mg-ATPase which is consistent with the physiological observations of muscle contraction in vivo. To strive toward this goal we have been employing the stopflow technique to study the elementary steps of the interaction between myosin active site and ATP in the presence or absence of actin. Furthermore, with the same technique we will be able to study this interaction in the presence of the regulatory proteins, troponins and tropomyosin. We also hope that from the knowledge we gain from the studies on skeletal muscle, we will be able to understand the mechanism of motility as a whole.

Methods Employed and Major Findings:

Using the stopflow technique we have been able to study the kinetics of protein substrate interaction in the millisecond time range. For the studies on the mechanism of myosin Mg-ATPase, we have employed the technique of monitoring (1) the H^+ changes by means of observing the O.D. changes of a pH indicator dye in the reacting solution and (2) the intrinsic tryptophan fluorescence changes during the course of the reaction initiated by adding ATP to myosin active site in the presence of excess magnesium. For the studies on the mechanism of actomyosin Mg-ATPase, we have employed the techniques of monitoring (1) the turbidity changes by means of measuring the light scattering of the reacting solution and (2) the intrinsic tryptophan fluorescence changes during the course of the reaction. The recent findings based on the above techniques are summarized as follows:

(A) Interaction of ATP with myosin, heavy meromyosin (HMM) and subfragment-1 (S-1).

Since myosin is not soluble below ionic strength of 0.5M KCl, most of the kinetic investigations on the mechanism of myosin and actomyosin ATPase have

been done using the more soluble proteolytic subfragments of myosin, HMM and S-1. The basic question, therefore, is whether, under the conditions where they can be compared, do myosin, HMM and S-1 behave identically with respect to their interaction with ATP. This question is answered by monitoring the protein fluorescence changes of the reaction initiated by adding ATP to myosin, HMM, or S-1 under various experimental conditions; our results show that they do behave similarly. Furthermore, for HMM, where H^+ release with respect to ATP hydrolysis has been studied, our results from the fluorescence studies suggest that the release of H^+ is concomittant with a protein conformational change. However, this result will have to be tested under more rigid conditions before a firm conclusion can be drawn. Finally, it was discovered that at low salt and low temperature, the rate of the protein conformational change following the binding of ATP to HMM or S-1 no longer obeys the hyperbolic relationship derived from the two-step binding mechanism, rather it levels off abruptly. This phenomenon cannot be explained using the two step reaction scheme. If this observation is correct, it suggests that either the two-step mechanism should be extended to include more intermediates or that at low salt and low temperature the interaction of ATP with myosin active site is via a different mechanism.

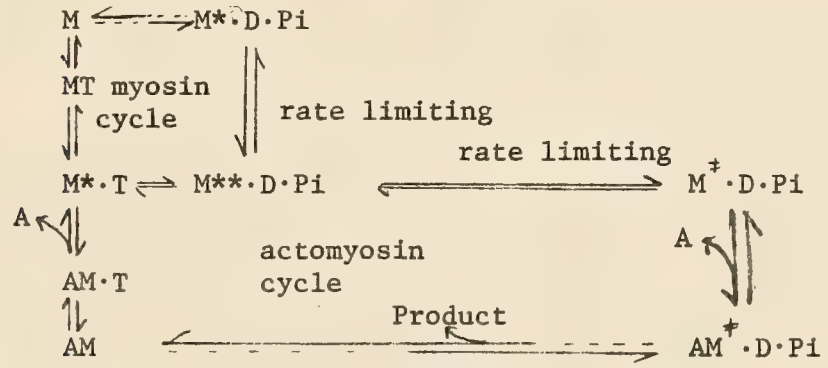
(B) Interaction of ATP with SH_1-blocked myosin subfragment-one

In collaboration with Dr. Sally Mulhern, we have studied the interaction between ATP and myosin subfragment-one blocked at the SH_1 sulfhydryl group with N-ethylmaleimide. Our results show that, as for normal S-1, the binding of ATP to NEM-S-1 follows a two-step mechanism involving first, the rapid formation of a collision intermediate followed by a slower conformational change causing an increase in fluorescence. We, therefore, could investigate the effects of salt and temperature on the equilibrium constant for the formation of the collision intermediate (K_a) and the rate of the conformational change (k_2). We found that increasing KCl from 0.1 to 0.5M increased K_a by two-fold, whereas with the unmodified S-1 it actually decreased it by ten-fold. Under the same conditions, the increase of salt resulted in a ten-fold decrease in k_2 whereas with unmodified S-1 it actually increased it by three-fold. As for the effect of temperature an increase from 10 to 20° had very little effect on the K_a of both the NEM and unmodified S-1. However, with unmodified S-1 the rate of the conformational change increased markedly with increasing temperature, i.e. the activation energy was quite high; whereas with NEM-S-1 this step showed a much lower temperature sensitivity. Therefore, blocking the SH_1 group not only quantitatively changed but in some cases actually reversed the effect of salt and temperature on the step leading to the formation of the collision intermediate and the subsequent step involved in the formation of the fluorescent intermediate.

(C) Interaction of actomyosin with ATP.

The utilization of the presteady state technique enables us to investigate the mechanism of actomyosin-ATP interaction during the hydrolysis of ATP using the single turnover technique. By monitoring the light scattering changes during the interaction of acto-S-1 with ATP it was found that under the condition of actin activation there is a cyclic interaction between S-1 and actin each time an ATP is hydrolyzed, i.e. acto-S-1 is quickly dissociated when ATP binds and reassociated after ATP is hydrolyzed.

Furthermore, the rate of the reassociation of S-1 to actin after the hydrolysis of ATP is equal to the steady state acto-S-1 ATPase both at low and high actin suggesting that acto-S-1 undergoes the same cyclic dissociation and reassociation each time an ATP is hydrolyzed. This observation supports the physiological postulate that, in order for muscle to contract, the crossbridges have to undergo a cycle consisting first [a] detachment of the crossbridges from the thin filament and then reattachment of the crossbridges to a different position along the thin filament during the hydrolysis of ATP. The fact that the rate of the reassociation of S-1 to actin levels off at high actin suggests that the rate limiting step in the actomyosin cycle is not product release, but rather is a step which occurs before the reassociation step. The rate limiting step has been postulated by Eisenberg and Kielley as the step involved in the transition of the S-1 from a "refractory state" which cannot bind actin to a "nonrefractory state" which can bind actin. By using the fluorescence technique, a new intermediate which might correspond to the "refractory state" in the actomyosin cycle was elucidated. The rate for the formation of this fluorescent intermediate is about ten times faster than the rate limiting step for the cycle and is about equal to the rate for the formation of the fluorescent intermediate in the myosin cycle. This observation, together with other observations especially by Bagshaw et al. on the mechanism of myosin ATPase, led us to postulate the following scheme as a tentative mechanism of actomyosin and myosin ATPase.



where M is myosin, AM is actomyosin, T is ATP, D is ADP, Pi is orthophosphate, and the asterisk and ‡ represent different conformational state of the myosin.

Proposed Course of Research.

At this time, the immediate question to be answered is what is the nature of the fluorescent intermediate into which actomyosin dissociates following the binding of ATP. It is still not certain whether the "M*·T" species in the actomyosin cycle could be a species different from the M*·T in the myosin cycle. And there is still a question whether there is a conformational change when S-1 binds to F-actin. The effect of turbidity on the amplitude of fluorescence observed would also have to be clarified. Secondly, the mechanism of SH-blocked myosin requires more investigation before a comparison can be made with unmodified myosin. This would require the studies on the nature of the rate limiting step and the characteristics of its interaction with actin. Finally the kinetics of ATP binding to myosin active site at

low salt and low temperature will have to be reinvestigated before any firm conclusion can be drawn regarding the mechanism of the binding of ATP. The use of computer curve fitting technique will be used to clarify the different kinetic models.

Publications:

Chock, S.P., Chock, P.B., and Eisenberg, E. Pre-steady state kinetic evidence for a cyclic interaction of myosin subfragment-one with actin during the hydrolysis of ATP. Biochemistry (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00412-01 LCB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Interaction between Acanthamoeba Actin and Rabbit Skeletal Muscle Tropomyosin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. Yeu-Zu Yang, Visiting Fellow, LCB, NHLI
Other: Evan Eisenberg, Head, CP, LCB, NHLI
David Gordon, Staff Associate, LCB, NHLI
Edward D. Korn, Chief, LCB, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
NHLI, NHI, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	1.1	0.25

SUMMARY OF WORK (200 words or less - underline keywords)

Using I^{125}-tropomyosin, we find that Acanthamoeba actin differs from muscle actin in its interaction with tropomyosin. At 5mM $MgCl_2$, tropomyosin fails to bind Acanthamoeba actin and does not inhibit the acto-HMM ATPase. However, the Acanthamoeba actin does contain tropomyosin binding sites because binding does occur when HMM or troponin-I is present. The presence of 80mM KCl and 5mM $MgCl_2$ also causes tropomyosin to bind but instead of inhibiting the acto-HMM ATPase, as occurs with muscle actin, the binding results in 2 to 3-fold activation in acto-HMM ATPase.

PHS-6040
(12-75)

Project Description:

Objectives:
Numerous reports in recent years have shown that contractile proteins similar to those of muscle are present as common cellular components in non-muscle cells. These proteins, such as actin and myosin, are implicated in such vital cellular processes as motility, cytoplasmic streaming, cytokinesis and morphological development. In order to understand these cytoplasmic proteins, Dr. Korn had initiated biochemical studies a few years ago with Acanthamoeba castellanii as a model organism. As an extension of these studies, this investigation was undertaken, to further characterize Acanthamoeba actin, with special emphasis on its interaction with muscle tropomyosin and troponin. These interactions are of particular interest because, in the case of muscle, the contraction mechanism is regulated through the interaction of tropomyosin and troponin with the actin filaments. Previous studies from this laboratory (Proj. No. Z01 HL 00408-03LCB) have established that, in the case of muscle, tropomyosin binds actin and partially prevents it from interacting with myosin and that the presence of troponin-I accentuates the binding and further inhibits its interaction with myosin. It is anticipated that the effects of tropomyosin and troponin on Acanthamoeba actin, compared with those on muscle actin, may reveal how Acanthamoeba actin functions -- which in turn may be used as a tool to shed some light on the mechanism of muscle contraction.

Methods Employed and Major Findings:

An improved method, developed by Dr. David Gordon, was employed for the isolation of Acanthamoeba actin in high yield (Proj. No. Z01HL 00501-02LCB). The binding of ^{125}I-labeled tropomyosin to Acanthamoeba actin which results in its sedimentation upon ultracentrifugation was used as one measure of actin-tropomyosin interaction. It is also known that in the presence of 5mM $MgCl_2$, 2mM ATP, and 1mM EGTA, tropomyosin binds to muscle actin in a ratio of 1 to 7 and inhibits the acto-HMM ATPase by about 60%. We now find that under the same conditions, the binding of ^{125}I-tropomyosin to Acanthamoeba actin is very weak and that tropomyosin does not inhibit the Acanthamoeba acto-HMM ATPase. However, Acanthamoeba actin does contain tropomyosin binding sites since, if ATP is deleted from the 5mM $MgCl_2$ medium, HMM causes tropomyosin to bind to Acanthamoeba actin in the same stroichiometry as to muscle actin. Preliminary studies show that troponin-I also strongly increases the binding of tropomyosin to Acanthamoeba actin. Furthermore, in the presence of 80mM KCl and 5mM $MgCl_2$, binding occurs between Acanthamoeba actin and tropomyosin even in the absence of HMM. However, under such conditions, tropomyosin stimulates the Acanthamoeba acto-HMM ATPase 2 to 3-fold rather than inhibiting it as occurs with muscle actin.

Preliminary experiments indicate that Acanthamoeba actin exhibits negative

cooperativity when copolymerized with muscle actin. The random presence of Acanthamoeba actin monomers in the hybrid polymer effectively blocks the binding of muscle actin and tropomyosin under conditions where interaction would occur for pure muscle actin. Therefore, Acanthamoeba actin and muscle actin differ in their interaction with tropomyosin with respect both to the conditions required for binding and the effect of the bound tropomyosin on the actin-activation of HMM ATPase.

Proposed Course of Work:
More satisfactory procedures need to be developed for the separation and purification of troponin into its three subunits. Then the effects of these subunits on Acanthamoeba actin, individually and in combinations, will be investigated in the presence and absence of tropomyosin. These studies will be extended to the "hybrid" actin composed of Acanthamoeba actin and muscle actin in the form of "addition" copolymers. Copolymers of more defined composition will be chosen to study the nature of the negative cooperativity exhibited by Acanthamoeba actin.

Publications:
None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00401-10LCB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Electron transport in E. coli and rat liver mitochondria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard W. Hendler, Head, ME, LCB, NHLI

Collaborators: A. Instrumentation
Tom Clem and Larry Thiebault of Electrical and Electronic Engineering Section of the Biomedical Engineering and Instrumentation Branch, Div. of Research Services.
B. Microcomputer Adaptation
David Songco, Computer Systems and Laboratory, Div. of Computer Research and Technology

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Membrane Enzymology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	

SUMMARY OF WORK (200 words or less - underline keywords)

Two pairs of miniaturized platinum and reference electrodes are set in a cap which tightly fits an optical cuvette. The oxidizing potential of a solution or suspension of respiratory components is measured and used to direct pulses of electricity through the second pair of electrodes, from a constant current generator, in order to change or maintain the oxidizing potential of the cuvette contents. The cuvette is placed in a split-beam dual-wavelength spectrophotometer so that the redox state of its cytochromes can be optically monitored. This experimental set-up is to be used for potentiometric titrations and for conducting and storing a series of spectral scans at fixed potentials. The entire system is integrated with and under the control of a microcomputer.

PHS-6040
(12-75)

Project Description:

In order to extend the usefulness of potentiometric techniques to a fuller study of respiratory components, a new approach was conceived whereby the redox potential of a solution would be manipulated and set by electric currents. Initial studies (Project Report Z01HL 00401-09LCB for 1975) demonstrated the feasibility of such an approach. This report describes further steps towards the development of this system.

A. Studies on the kine of electrical source needed.
The influence of the power source on the potential of the solution and the redox state of either ferricyanide, cyt c or cyt b_1 was examined. The first experiments were performed using constant voltage from either batteries or electronic circuits. Variations tried, included continuous titrations with the voltage manually increased as necessary, pulses of different lengths applied at set voltages, and pulses of set lengths with different voltages. Next, experiments were conducted with a constant current generator which automatically altered voltage as needed to maintain a selected current. The variations of continuous titration vs pulses with changes in current settings were also tried.

B. Feed-back control systems.
The use of the measured solution potential to control both constant voltage and constant current generators was investigated. When the solution potential varied beyond set limits power was applied to maintain a desired potential. The same principle was used to construct a system for automatic titration whereby an electronic voltage ramp continuously changed the value of the desired solution potential and the feed-back control system reacted to maintain solution potential equal to the ramp voltage. Similar experiments were performed with a ramp of continuously changing currents in the current-controlled system.

As a result of these studies we decided to adopt the controlled current approach. This decision was based on the fact that the internal electrical resistance due to the electrodes and solution components dramatically changed during the titration so that the amound of electrochemical oxidation or reduction per unit amount of applied voltage markedly varied.

Furthermore the controlled voltage system caused continuous fluctuations in the actual sign of the voltage on the generating electrode. Because cytochromes tended to react more quickly with the electrode then did the overall redox state of the system, wide swings in the redox state of the cytochromes were observed to accompany the feedback-control process. Electronic damping and non-linear amplifiers did not resolve this problem.

C. Other factors studied.
Factors which could influence the response of the system were investigated such as the nature of the salts and their concentrations, the area of the generating platinum electrode, and the effects of soluble redox mediators.

As a result of these studies, a mixed salt system of KPO_4 and KCl was adopted. The area of the generating electrode was maximized and a system of redox mediators chosen. A particularly important factor studied was that of mixing. As a result, we decided to make the generating electrode the solution mixer. A perforated platinum sheet turned into a cylinder, with internal paddles, was constructed. This mixing electrode, housed in a bushing with lubricated sleeve bearings was set into a molded silicone cap and oscillated by an electric motor.

D. Overall findings with the above system.
It soon became obvious that continuous titrations could not be accomplished because of field effects generated by the working electrodes on the measuring electrodes and because the system was continuously pushed out of redox equilibirum by the incoming electricity. A series of pulses and pauses, however, did lead to titration curves for ferricyanide, soluble cytochrome c, and membrane-bound cytochrome b_1. However, the curves were somewhat distorted compared to theoretical and traditionally obtained titration curves. Furthermore, successive oxidative and reductive titrations of the same substance most often resulted in hysteresis effects in parts or all of the curves.

E. Limitations on further development with analogue circuitry.
All of the developments so far discussed were carried out with circuits specifically designed for particular tasks. A major problem was discovered in the operation of the system being developed. It was found that after a pulse of electricity, the generating electrode remained polarized so that during the pauses allowed for system equilibration, the 'resting' generating electrode continued to generate new redox species and thus retard or prevent complete equilibration. The residual charge left on the generating electrode could be increased, decreased or reversed in sign depending on the nature of the pulse used. This observation suggested a way to resolve the problem. The measuring electrode could be used to determine the magnitude of polarized potential left on the 'resting' generating electrode. The difference in voltage between the measuring electrode (i.e., the solution potential) and the generating electrode (i.e., amount of polarization) could be used to send very short correcting pulses through the generating electrode to remove its charge. This increase in complexity of circuit demand is too great to be adequately handled by analogue circuitry. A micro computer could easily do this as well as continuously monitor optical densities (i.e., redox state of the cytochromes), solution potentials, and generating voltages to see that the system is always in equilibrium when data measurements are taken.

In addition to performing potentiometric titrations throughout broader voltage regions than are possible with classical chemical systems, the micro computer controlled system can be used for attaining and holding a series of fixed potentials, performing spectral scans, storing families of scans in core, and generating difference spectra for different sets of oxidizing potentials.

F. Progress made with the microcomputer-controlled system.
An "Intel microporcessor" has been purchased and suitable A/D and D/A interfaces are being constructed, as well as the microcomputer-controlled current generator. In the meantime, I have learned the languages necessary to program the microcomputer. I have written the software packages necessary to carry out the two kines of experiments (e.g., potentiometric titrations and spectral scans at a series of fixed voltages). I have collaborated on the development of a software communications package so that the microcomputer can obtain stored programs directly from the in-house computed (PDP-10) and can send data, accumulated from experiments, back to the PDP-10 for storage and manipulation.

Proposed Course:

We will shortly test the composite system consisting of microcomputer, hardware interfaces, accessory devices, and software, under simulated experimental conditions and then begin experiments of the two kinds described in this report. As soon as the system is made functional, it will be used to study further the respiratory chains of E. coli and rat liver.

Publications:

Heindler, R.W. Respiration and energy transduction in Escherichia coli in membrane transport, volume 3 (ed. A.N. Martinosi), Plenum Press, N. Y. (in press).

Project Description:

We have described a DNA-synthesizing complex, isolated from E. coli, which contains DNA polymerase I and presumably the recBC enzyme (exonuclease V). The complex is tightly bound to DNA and is released by thorough digestion of the DNA with pancreatic DNase (DNase I). In order to establish more rigorously the identity of the complex we pursued two simultaneous approaches. On the one hand, conditions were sought for the synthesis of the complex from purified DNA polymerase I and exonuclease V. On the other hand, we tried to improve the purification of biological complex so that we could identify the enzymes present and also compare it to synthetic complex.

Major Findings:

Attempts to separate biological complex from free exonuclease V, based on the ATP-binding properties of both, were made using affinity columns of ATP bound to Agarose. Two kinds of columns were tried - one in which ATP was bound through its ribose and another where ATP was bound through the N6 position of the purine. Variations tried included stepwise and continuous gradients for ATP, Mg^{++}, salt, DNA, and EDTA. In general, exonuclease V and complex had very similar affinities for both columns. Under conditions for binding, both enzymes were subsequently eluted together or only slightly separated. Recoveries of polymerase activity were low.

Preliminary experiments to synthesize complex from DNA polymerase I and exonuclease V indicated that the presence of native DNA was needed. In order to release any newly formed complex, total digestion of the DNA would then be required. With the aim of reducing the number of components present during this synthesis procedure, experiments were tried to accomplish the digestion of DNA with the nuclease activities of the polymerase and the exonuclease without the further addition of pancreatic DNase. However, the combined activities of the two enzymes resulted in the hydrolysis of only about 1/3 of the DNA. It was concluded that the action of pancreatic DNase was required to release products from association with DNA. The initial criterion for formation of a complex from DNA polymerase I and exonuclease V was the de-novo appearance of ATP-stimulated polymerase activity in an elution volume from Biogel A1.5M corresponding to a heavier molecular weight entity than DNA polymerase I. When the formation of synthetic complex was attempted without the use of pancreatic DNase, 13% of the applied polymerase activity met the criterion for complex formation. Twenty-three percent was found in the void volume and 58% was found in the low molecular weight fractions from the BioGel column. In all other experiments, pancreatic DNase was employed. When the DNase was added after the polymerase and exonuclease were incubated with DNA or even after only the polymerase was incubated with DNA, 70 to 80% of applied polymerase activity met the criterion for complex formation. Digestion of the DNA prior to addition of the polymerase and exonuclease reduced the apparent yield of complex to 35% and the use of the two component enzymes without DNA and DNase reduced the amount to 15%. In all cases in which DNase was present, there was a huge peak for free polymerase activity resulting in substantially greater recovery of activity than was applied. This is because the tailing of DNase into

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fractions containing the polymerase resulted in the activation of the native DNA as a template. Incubations of polymerase, exonuclease V, and DNase in the absence of DNA yielded about 5% of the activity in the high molecular weight region with erratic ATP-stimulations.

Proposed Course of Research:

The formation of complex from purified enzymes will be pursued. Characterization of the nature of the association between DNA polymerase and exonuclease V will be a major effort as well as the comparison of synthetic and native complexes.

Publications:

Hendler, R.W., Pereira, M., and Scharff, R.: DNA synthesis involving a complexed form of DNA polymerase I in E. coli extracts. Proc. Nat. Acad. Sci. U.S.A. 72: 2099-2103, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00411-01 LCB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Reconstitution of E. coli succinoxidase system from sub-components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard W. Hendler, Head, ME, LCB, NHLI
Other: Tirumura L. Reddy, Visiting Scientist, ME, LCB, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Membrane Enzymology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

SUMMARY OF WORK (200 words or less - underline keywords)

E. coli membranes are extracted first with 0.4% deoxycholate (DOC) and then with 2% DOC to yield crude preparations of succinate dehydrogenase and cytochrome oxidase. These fractions, when passed together through Sephadex G-75 to remove DOC, regain succinoxidase activity and a particulate structure. When the fraction are individually passed through Sepharose 4B or Bio Gel A 0.1M they lose a significant fraction of their reconstitutive ability. A soluble fraction has been isolated which can restore the reconstitutive ability of the column chromatographed fractions. The soluble factor is heat stable, is not succinate dehydrogenase or cytochrome oxidase, is not ^{55}Fe labeled but does appear to contain phospholipid.

PHS-6040
(12-75)

Project Description:

Background:

In previous work, we have solubilized E. coli membranes in deoxycholate (DOC and separated the released electron transport chain into fractions containing dehydrogenases and/or cytochromes. In this project we are trying to reform both a membrane structure and an integrated succinoxidase respiratory chain from the separated non-membranous fractions. The initial approach involves two crude fractions: 1) A 0.4% DOC extract containing nearly all of the succinate dehydrogenase with associated cytochrome b_1, a non-heme iron component, and only a few percent of the original cytochrome oxidase. 2) A 2% DOC extract of the 0.4% DOC residue (2-0.4% extract) containing about half of the original cytochrome oxidase, about 1/3 of the original cytochrome b_1, and only a few percent of the original succinate dehydrogenase.

A. Reconstitution of a succinoxidase chain from crude succinate dehydrogenase (SDH) and oxidase fractions.

DOC was found to be very destructive to the reconstitutive ability of extracted SDH. However if the time of exposure of SDH to DOC was kept to a minimum, 2% DOC was the optimal level of bile salt for extracting reconstitutively-active SDH and oxidase from the membranes. Reconstitution, as measured by the appearance of succinoxidase activity, could be accomplished by extensive dilution of the combined DOC extracts followed by centrifugation to harvest the membranes. A superior method, however, was to pass the combined extracts through a small column of Sephadex G-75 which yielded the reconstituted material in the void volume and DOC in the included volume. The level of reconstituted succinoxidase was at least equal to that in the original membranes. However, because of the cross-contamination of the dehydrogenase and oxidase fractions, each possessed endogenous activity and the level of reconstitutive activity was only 2 to 3 times that of the sum of the two endogenous activities.

B. Attempts to reduce levels of endogenous succinoxidase activities and discovery of a reconstruction factor.

Passage of the crude SDH and oxidase fractions over Sepharose 4B in order to lessen the degree of cross contamination led to SDH and oxidase fractions which were reconstitutively inactive with each other and with the complimentary crude fraction. Suspecting that some essential small molecule(s?) were being lost during chromatography on Sepharose 4B, the crude oxidase fraction was simply filtered on an Amicon, XM300 membrane. This procedure lowered the endogenous activity of the oxidase fraction and also markedly lowered the

extent of reconstitution of succinoxidase activity. Adding XM300 filtrate to the reconstitution mixture increased the level of obtained succinoxidase activity by several fold. The concentrated factor from the XM300 filtrate was colorless and contained an insignificant amount of SDH and no cytochrome oxidase.

C. Properties of the factor.
A titration of SDH and oxidase with increasing amounts of factor led to two kinds of curves. With certain preparations a sharp peak of optimal factor concentration was seen (i.e. excess factor inhibited). With other preparations a saturation curve was obtained. The factor is stable to 10 minutes heating at 80°C. When passed through Sepharose 4B, Bio Gel A 0.5m, Sephadex G-150 and Sephadex G-100, three peaks of activity are obtained covering a molecular weight range of about 20,000 to 150,000. Factor was isolated from preparations with ^{55}Fe-labeled cytochromes and ^{3}H-labeled phospholipid. It appears that ^{55}Fe does not co-chromatograph with factor activity but phospholipid does co-chromatograph with the largest of the three species.

D. Controls for reconstitution.
A crucial question is whether separate vesicles enriched with SDH react with vesicles containing oxidase or whether both activities have become incorporated in the same vesicle. If two separate populations of vesicles exist, the concentration of each would be decreased by dilution and the frequency of fortuitous collisions of SDH and oxidase should be decreased by the square of the dilution factor. If both activities are in the same vesicle,activity should be linearly related to dilution. We find that the activity of diluted preparations is at least as high (or higher) than that expected from a linear relationship. Furthermore, when separate preparations of DOC-free SDH and oxidase were mixed together, no increase in succinoxidase activity was noted.

Cyanide inhibits succinoxidase by inhibiting the interaction of cytochrome oxidase with oxygen. If electrons from succinate, in the reconstituted preparation, travel through a complete electron transport chain to oxygen, then the cyanide sensitivity of the reconstituted preparation should be as great as that of the original membranes. This was found to be the case.

E. Other findings.
The endogenous succinoxidase activities of both SDH and oxidase preparations are considerably lowered by passage through Bio Gel A 0.5m. Using these preparations plus added factor, a 10 fold increase in succinoxidase activity can be obtained during reconstitution.

Mg^{++} has proven to be rather important in reconstitution. It increases the quantity of reformed membranes and raises the level of reconstituted succinoxidase by about 50%.

Proposed Course:
A collaboration with Dr. Blair Bowers to perform electron microscopy on thin

sections as well as by negative staining and with Dr. Pinto Dasilva to perform freeze cleavage studies by electron microscopy has been established. We shall try to determine whether membrane structures are formed during the process of reconstitution of the succinoxidase system and whether any unique arrangement of intramembranous granules can be used to identify the succinoxidase system in the membrane. We shall also determine biochmically whether the Ca-Mg activated ATPase is incorporated into the membranes during reconstitution and whether reconstituted membranes are capable of energy transduction.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 0503-04 LCB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure, Assembly and Function of Microtubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Flavin	Head, Sect. Organelle Biochem.	LCB NHLI
	Y. Nagata	Visiting Fellow	LCB NHLI
	D. Raybin	Staff Associate	LCB NHLI
	K. Summers	Staff Fellow	LCB NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Organelle Biochemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8	8	0

SUMMARY OF WORK (200 words or less - underline keywords)

Our research deals with the structure, assembly and function of microtubules, an organelle involved in chromosome segregation, cell motility, and the maintenance of cell shape. In the past year we have been studying a unique post-translational modification of tubulin (the protein building block of microtubules) which we think is an excellent candidate to regulate microtubule assembly or function. This involves the reversible enzymatic addition of a tyrosine residue to the C-terminus of the α-chain of tubulin. We are also studying the role of a calcium-specific ATPase as an auxilliary energy transducer or a component of the steering apparatus in Chlamydomonas flagella. A third project concerns the identification of a specific inhibition of dynein, the primary energy transducer in cilia and flagella.

PHS-6040
(12-75)

Project Description:

Objectives: Microtubules, found in the cytoplasm and dividing nuclei of all eukaryotic cells, are formed by the polymerization of a dimeric precursor (tubulin) consisting of 2 very similar 55,000 dalton subunits, called α and β chains. Polymerization is induced by warming suitable brain extracts, is reversed by cooling, and tubulin can be purified by repeated cycles of warming and cooling. Usually small amounts of certain other proteins copolymerize. Since in living cells microtubules may form and dissolve rapidly when the total content of tubulin appears constant, it seems unlikely that transcriptional and translational controls could be sufficient to regulate microtubule assembly *in vivo*. Reversible post-translational modifications of tubulin are therefore intriguing candidates to modulate the assembly (or function) of microtubules. One example is the binding, and certain transformations (which we described in last year's report), of guanine nucleotides. Another is the phosphorylation of serine residues in the β chain. However, no evidence has been reported that the phosphorylation can be reversed. We have been studying a third kind of post-translational modification of tubulin, which appears to be quite unprecedented. This project stems from the discovery, in Caputto's laboratory in Argentina, that rat brain extracts could incorporate tyrosine into protein in the absence of tRNA or any other component of protein synthesis. Protease digestion suggested that the tyrosine was incorporated into the C-terminal position of a protein which was noted to have a number of similarities to tubulin.

Flagellar motility is mediated by energy-transducing protein(s) called dynein, located at least in part in arms attached to the outer doublet microtubules. Dynein can be solubilized as a heterogeneous, high molecular weight ATPase, activated by either Mg or Ca. We had previously found that *Chlamydomonas* flagella contain, in addition to dynein, a low molecular weight Ca-specific ATPase. The principal possible functions for this enzyme would be as an auxiliary energy transducer, possibly in the spoke head structures, or in steering or tactic responses, in which Ca and ATP have been implicated.

Major Findings:

A. Microtubule Assembly

1) Post-translational addition of tyrosine to microtubule protein (D. Raybin): Last year we described the isolation, from brain extract, of an enzyme which adds a tyrosine residue to the C-terminus of the α chain of tubulin, apparently through peptide bond linkage to a glutamate or glutamine residue. Tubulin purified by 3 cycles of *in vitro* assembly was free of, and could be used to assay, the enzyme, and the enzyme (tentatively called tubulin-tyrosine ligase) was easily obtained free of native tubulin. Crude enzyme was able to incorporate at least 0.25 mole of ^{14}C-tyrosine per mole α chain in tubulin isolated by 3 cycles of assembly, and the radioactivity thus incorporated could then copolymerize with added purified tubulin through several additional cycles of assembly.

a) Properties of tubulin-tyrosine ligase: The enzyme has been purified 40 fold from bovine brain, in poor yield (see below). It was present in the 100,000 x g supernatant fraction of every rat tissue examined. The highest specific activity was found in extracts of cultured, undifferentiated neuroblastoma cells. In all extracts except brain, activity was only detectable after adding purified brain tubulin; the other tissues did not contain detectable levels of tubulin competent to serve as a substrate. Ligase has not been detected in extracts of the microtubule-containing unicellular eukaryotes: yeast, Tetrahymena and Euglena. However, the latter contain a protein component which can enhance brain ligase activity (see below). The ligase was also absent from extracts of cilia prepared from Tetrahymena.

The enzyme was specific for the α chain of tubulin: no ^{14}C-tyrosine was fixed into any other brain component, nor when purified enzyme was incubated with large amounts of pure proteins, other than tubulin, containing C-terminal glu (avidin, horse heart cytochrome C) or gln (A-1 lipoprotein, micrococcal nuclease). Among other free amino acids, only L-phenylalanine and L-dopa had been shown able to react in place of L-tyrosine, the respective K_m values being 3000, 300 and 30 μM. Structurally related neurotransmitters were not inhibitors (or substrates), i.e. dopamine, epinephrine, norepinephrine, octopamine and serotonin. However, some other tyrosine derivatives were competitive inhibitors: L-tyr-L-tyr (K_i 25 μM), L-glu-L-tyr (K_i 150 μM), tyramine (K_i 2000 μM).

Tyrosylation of tubulin requires the addition only of ATP, Mg^{+2} and K^+. To study the reversibility of the reaction, substrate was prepared by incubating tubulin with purified ligase and ^{14}C-tyrosine, and isolated by 3 cycles of assembly. Free tyrosine was liberated again in the presence of ADP + P_i or arsenate, and comparison of rates with crude and purified ligase fractions indicated that addition and removal of tyrosine were catalyzed by the same enzyme. The rate of detyrosylation was markedly dependent on the proportion of ^{14}C-tyrosine (from 1 to 5%) that had been initially introduced, suggesting that brain tubulin as we isolate it is not already extensively tyrosylated. However, this important question has not been settled (see below).

Brain ligase was stable at -80° but was more heat sensitive than tubulin (90% denatured in 2 minutes at 52°). GTP could replace ATP but less effectively; cAMP or cGMP added in addition to ATP had no effect on the rate of tyrosylation. Preliminary studies on the subcellular localization of the enzyme in rat liver indicate it is absent from the lysosomal and microsomal fractions. Other organelles have not been examined, but the enzyme was found in the 100,000 x g supernatant of all rat organs after brief Teflon-glass homogenization.

b) What kinds of tubulin are substrates for the tyrosine ligase?: No tyrosine was fixed when brain enzyme was incubated with Tetrahymena cilia demembranated with Nonidet P40, or with isolated intact outer doublet microtubules or solubilized central pair tubulin. Three molecular species of

cytoplasmic tubulin have been characterized in brain extracts: 6s dimeric tubulin composed of 1 α and 1 β chain, 36s ring structures, and microtubules. The latter 2 structures are composed primarily of tubulin, with smaller amounts of several as yet poorly characterized associated proteins. The 6s dimeric tubulin isolated from an agarose 15m column was a good substrate for the ligase; since this tubulin is incompetent to form rings or microtubules we can conclude unequivocally that it is a substrate. It has not been proven whether tubulin incorporated into rings or microtubules can also accept tyrosine. Native α chains have not been shown to be in equilibrium with tubulin dimers, and it seems unlikely that α chains are a substrate or that tyrosylation is required for monomer → dimer. It is conceivable, although most unlikely, that the C-terminal tyrosine is a coded residue, and the initial post-translational modification is its removal through the reversible reaction catalyzed by the ligase. In this connection we find that tyrosine addition to α chains by intact cultured neuroblastoma cells is inhibited under conditions permitting protein synthesis, and enhanced when the latter is inhibited.

When ^{14}C-tyrosylated tubulin, purified by 3 cycles of assembly, was depolymerized by chilling and fractionated into 6s and 36s components with an agarose column, ^{14}C partitioned indiscriminately between the 2 fractions, suggesting that tyrosylation is unrelated to the formation of 36s ring structures from tubulin dimers.

c) Tyrosylation and microtubule assembly: The most obvious and immediate question is how the addition of tyrosine affects the competence of tubulin to assemble *in vitro*. When 3-cycle purified tubulin was tyrosylated and polymerized 3 more times, there was no significant enrichment of ^{14}C in tubulin which did or did not polymerize. Clearly C-terminal tyrosine did not block incorporation of tubulin into microtubules. Can tubulin assemble without C-terminal tyrosine, and to what extent is tubulin already tyrosylated as we isolate it? Pending an answer to the latter question, we tried to prepare completely detyrosylated tubulin, by incubation with purified ligase and ADP + P_i, and then see whether it would assemble. In order to show that it had been completely detyrosylated, it was necessary to start with tubulin containing a small proportion of ^{14}C-tyrosine. This approach has been unsuccessful due to a) the kinetic behaviour of the reverse ligase reaction, mentioned above, and b) the fact that tubulin assembled poorly after incubation with large amounts of purified ligase, for unknown reasons unrelated to extent of tyrosylation.

The proportion of tubulin, as isolated, which is already tyrosylated would be most easily determined if one could physically separate the tyrosylated from the non-tyrosylated α chains. Brain α chain had recently been resolved into 2 components with SDS-hydroxylapatite columns, but we found that the radioactivity of tyrosylated tubulin partitioned indiscriminately between the 2 components. Electrophoretic fractionations reputed to resolve denatured α chain into as many as 6 components are under investigation. Meantime, pending

sequencing of α chain which is in progress in other laboratories, we have tried to study the C terminal residues in the intact chain with carboxypeptidase. Caputto's laboratory had shown that when a crude brain extract was subjected to partial acid hydrolysis after ^{14}C-tyrosine fixation, the only detectable radioactive dipeptide was α-glutamyl-tyrosine, indicating the penultimate residue in the α chain to be glu or gln. Eipper had identified a phosphorylated tryptic peptide from the β chain as being C-terminal since it lacked lys or arg. The peptide was obtained from a mixture of α + β chains, and from the total number of tryptic peptides it seemed likely that the α chain C-terminal peptide was probably quite homologous and was co-chromatographing with the phosphopeptide. The Eipper peptide(s) were very acidic and contained 1.8 tyrosines in a total of 50 amino acids.

Carboxypeptidase A rapidly released the ^{14}C from tubulin that had been tyrosylated with the ligase. All the ^{14}C was released from native tubulin; after performic oxidation 20% appeared resistant. Performic oxidized tubulin isolated by 3 cycles of assembly, and which had not been tyrosylated with the ligase, was treated with 500 times more carboxypeptidase, and the liberated amino acids were measured with a Durrum analyzer. No significant amount of tyrosine (0.06 mole/mole α chain) was detected. There was also no significant amount of glu or gln; however glu is known to be resistant, and we have not been able to recover C-terminal glu from cytochrome C with carboxypeptidases A or C. These results suggest that brain tubulin as isolated is not extensively tyrosylated. It should be noted that available information on conditions in vivo indicates that brain tubulin could be mostly tyrosylated since enzyme is in excess over tubulin in extracts, and the concentration of free tyrosine in brain exceeds the K_m concentration for the ligase.

On the other hand studies of the extent to which purified brain tubulin can accept additional ^{14}C-tyrosine indicate that not more than 25% of the α chains can do so. This was first thought to be a minimal value reflecting tubulin denaturation during the incubation with ligase, but experiments with 15 times higher concentrations of purified ligase have given the same result. Since 3/4 of isolated brain tubulin appears blocked towards accepting ^{14}C-tyrosine, and this is not due to prior presence of unlabeled tyrosine, the C-terminus of these α chains might be occupied by a moiety distinct from but related to tyrosine. We have therefore begun to study post-translational modification of tubulin in intact cultured neuroblastoma cells. Tyrosine fixation was much enhanced when protein synthesis was inhibited and was exclusively into the α chain of tubulin; 90% of the ^{14}C fixed appeared to be in an insoluble fraction of tubulin. We plan to isolate α chains from these cells and identify the labeled component(s) released by pronase, to see if anything other than tyrosine is present after proteolytic digestion.

d) Purification and subunit structure of tubulin-tyrosine ligase: The experiments described above have been done with ligase purified 40 fold by ammonium sulfate precipitation followed by elution from DEAE-Sephadex, but in only about 10% yield. A variety of procedures tried for the first step have all resulted in loss of activity, although the enzyme is very

stable to storage in crude extracts. Dialysis for 3 hours also caused a 60% loss of activity. Moreover the enzyme properties were altered by all precipitation procedures tried, since subsequently a portion of the enzyme activity could not again be dissolved. A possible basis for these difficulties has been revealed by studying the enzyme molecular weight with calibrated Sephadex G200 columns. The molecular weight was found to be 150,000 in extracts, but 70,000 after the above purification. When equal units of DEAE fraction (70,000) and fresh crude extract (150,000) were mixed just before applying to the column, all the activity eluted in the 150,000 fraction and the total units recovered were 2.5 times greater than before mixing. The enhancement of activity of the DEAE fraction was not observed with extract heated at 100°, and was partially reduced after 60° (which completely destroyed the extract ligase).

Extracts of yeast and Tetrahymena, which have no ligase activity, also enhanced ligase activity 2 to 3 fold when added to dialyzed brain extract or DEAE fraction. In the latter case, however, the molecular weight of the activity remained in the 70,000 fraction. The factor from Tetrahymena could be quantitatively assayed, and was in the macromolecular fraction from a molecular sieving column. These results indicate that the functional ligase can exist in different molecular forms, and suggest the existence of a regulatory subunit.

2) Direct observation of intermediates in microtubule assembly by darkfield light microscopy (K. Summers): With the aid of an intense light source and an image intensifier, darkfield microscopy has indicated the existence of sheet-like intermediate forms during microtubule assembly in vitro. By spraying the suspension onto grids and using special fixatives, some electron microscopic confirmation has been obtained, but the sheet forms were very labile and were often not seen. We are currently trying to improve fixation of these sheets by a rapid freeze technique.

Cytoplasmic microtubules are determinants of cell shape, and thus provide an approach to cell differentiation. Lionel Jaffe of Purdue University, who is interested in electrical controls of development, visited the laboratory to determine whether darkfield microscopy could be used to study the possibility that microtubules assembled in vitro will orient parallel to an electric field.

B. Flagellar Motility

1) Specific inhibitor of dynein (Y. Nagata): A specific inhibitor of dynein, the energy-transducing protein(s) of eukaryotic cilia and flagella, would have many applications, such as in investigating whether dynein or myosin were involved in various cases of cytoplasmic motility. Solubilized dynein has an ATPase activity activated almost equally well by Mg^{+2} or Ca^{+2}. In isolated demembranated flagella motility is induced only by Mg^{+2}. Last year we reported that, with one commercial preparation of ATP, solubilized Chlamydomonas dynein was activated normally by Ca^{+2}, but the Mg-ATPase was reduced by 80%. The inhibitory preparation had lower amounts of many metals

including Ca^{+2}; the latter added in addition to Mg^{+2} did not reverse the inhibition. It had a higher concentration of iron.

The inhibitor has now been shown to be consistently present in 3 different lot numbers of the ATP preparation, which is isolated from equine muscle, and to inhibit dynein from different sources, i.e. both 12s and 20s species from Tetrahymena cilia (species which separate in sucrose gradients and whose relation to each other is still obscure). There was no inhibition of muscle or cytoplasmic myosin ATPase, of the residual particulate ATPase after extraction of dynein from cilia, or of the soluble 4s Mg-ATPase of Tetrahymena cilia (see below), and ferrous or ferric iron (0.5 mM) did not inhibit. The inhibitor could be quantitatively assayed. It was retained by anion exchangers, and was bound weakly but a little more than ATP to cation exchangers, suggesting that it has zwitterion properties. The inhibitor was completely separated from ATP on molecular sieve columns; Sephadex G25 gave a wider separation than G10, G15 or G50. It appeared to be a smaller molecule than ATP. Experiments are in progress to isolate large amounts for structure determination.

2) Flagellar ATPases: We have previously reported that Chlamydomonas flagella (but not Tetrahymena cilia) contain, in addition to dynein, a distinct low-molecular weight Ca^{+2} specific ATPase. Little progress was made in the last year in determining the function of this enzyme.

Y. Nagata has found that some extracts of Tetrahymena cilia contain large amounts of a small (4s) ATPase which is specific for Mg^{+2}, but otherwise has some similarity to the Chlamydomonas enzyme in its size and lack of specificity towards other nucleoside triphosphates. ATPases were examined in extracts of cilia and flagella from various marine invertebrates collected by M. Flavin at Friday Harbor Laboratories. Although the results were not conclusive no evidence was found for a low-molecular weight Ca-ATPase in extracts of sperm flagella from Pisaster ochraceus, Strongylocentrotus franciscanus or purpuratus, or gill cilia from Clinocardium nuttali or Mytilus californianus. The sea urchin sperm tails were found to contain adenylate kinase, but unlike Tetrahymena and Chlamydomonas (which can regenerate amputated flagella) did not contain nucleoside diphosphokinase.

Our previous attempts to localize the Chlamydomonas Ca-ATPase had been unsuccessful. Fortunately George Witman of Princeton University visited the laboratory, and by fractionation procedures he had developed was able to show that Ca^{+2}-specific ATPase was located partially in the flagellar membrane and partially in the axoneme. It seems likely that there are either 2 different enzymes, or that 1 enzyme forms a structure bridging membrane and axoneme.

K. Summers could not confirm the previously suspected absence of 3s Ca-ATPase from a paralyzed mutant of Chlamydomonas. Recently in collaboration with G. Witman he has been studing mutant pf-14, which has a defect in the radial spokes. There is indirect evidence that the spoke heads should contain an energy transducing protein distinct from the dynein present in

the outer doublet arms. Although pf-14 flagellar extracts contain both the 12s and the 18s species of dynein characteristic of the wild type, the 18s dynein appears to be more labile or less securely anchored to axonemal structures.

Proposed Course of Research: The specific enzymic tyrosylation of tubulin interests us because, as a unique kind of post-translational modification, we feel it is an outstanding candidate to control microtubule assembly or function and in turn in influence cell division, differentiation and motility. Since the enzyme causing the modifications must itself be controlled to serve this purpose, it is encouraging that we now have some evidence for a regulatory subunit. Studies of the tubulin-tyrosine ligase are now developing rapidly enough that the immediate course of action is self-evident from the results described above. The enzyme must be further purified and the subunit structure characterized. We urgently need a method to determine what (tyrosine or an unknown related moiety) and how much is present at the C-terminus of cellular α-chains as isolated. This will eventually be revealed by complete sequencing. Meantime other possible approaches are physical separation of the modified chains, comparison of small C-terminal peptides after sequential protease treatment, and identification of radioactive components in tubulin modified in vivo. We do not know the stoichiometry of the enzyme reaction in vitro, and must confirm that tyrosine introduced is indeed C-terminal and in peptide linkage. We have not answered the immediate question of whether unmodified tubulin can polymerize normally in vitro. One would like to know the competence to polymerize, and capacity to accept tyrosine, of cell tubulin after treatment with carboxypeptidase.

Even if tubulin assembled indifferently with or without tyrosine in vitro, it is likely that the requirements would be more stringent in vivo, where many other conditions would not be optimized. In cells where assembly can be rapidly induced and reversed in the absence of protein synthesis (cultured fibroblasts, heliozoa, marine eggs, melanophores, etc.) we plan to compare the extent of tyrosylation in the 2 states. Microtubule assembly or function could also be controlled by tubulin modifications which altered its affinity for microtubule-associated proteins, or its partition among different cellular compartments. In this respect we have begun by studying the non-tubule, membrane bound moiety of tubulin in brain and other tissues. We have isolated this species of tubulin in soluble form, and plan to see if it is a substrate for the ligase, and whether the ligase can put it on or take it off the membrane. We have also begun to study the binding of cytoplasmic tubulin to membranes, by looking for energy transfer with different fluorescent labels in the 2 components, to see whether tyrosylation affects the binding.

It has been mutually beneficial to continue a project relating to axonemal microtubules while studying cytoplasmic microtubules. Clues to the function of the 3s Ca-ATPase in Chlamydomonas flagella should result from determining where it is located. We should now quickly be able to determine whether the membrane and axoneme-associated moieties of this activity reside in the same

or different proteins. Beyond that the project on flagellar motility is not now well defined. It is possible that, rather than pursuing the function of this flagellar enzyme directly, we would try to approach the so far neglected area of the Ca-responsive steering apparatus in cilia and flagella. Tactic responses in many cells require Ca, and phototactic mutants of Chlamydomonas are easily obtained. One would like to screen out sensory receptor and membrane-defective mutants, and score for abnormal Ca responses in ATP-reactivated isolated flagella. However, the great majority of "behavioural" mutants in Paramecium have proved to have ion-gate membrane defects. For biochemistry there is no obvious path. One could look for homologies to the muscle troponins or for Ca binding proteins (reported in sperm). Other possible points of departure might be the role of vitamin E in sperm motility, γ-carboxyglutamate, and some reported effects of nickel ions (we have previously looked for nickel-resistant mutants).

Prior projects not pursued in the past year are tubulin-bound nucleotides and their role in microtubule assembly, and drug resistant mutants of Chlamydomonas and related studies of podophyllotoxin chemistry. Virtually nothing has been done with dynein biochemically. The interesting evidence that microtubules can be stabilized by covalent cross-linking of dimers (Luduena) suggests a search for cross-linking enzymes. New methods for chemical dissection of axonemes would be useful, i.e. to solubilize native dimers from doublet microtubules (cells can do it but so far we can not), or to solubilize spokes or central sheath but not outer doublet arms.

Publications:

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ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART AND LUNG INSTITUTE

July 1, 1975 through June 30, 1976

Research in the Laboratory of Cellular Metabolism has been concentrated in four main areas. These are (1) the control of cyclic nucleotide phosphodiesterase and adenylate cyclase activities, (2) the metabolism and functions of cyclic GMP in arterial smooth muscle, leukocytes and lung, (3) the mechanisms through which histamine release from mast cells is initiated and terminated, and (4) the control of lipid synthesis, particularly cholesterol synthesis, in mammalian cells. Work on the regulation of hormone-sensitive lipase activity in adipose tissue has been suspended for most of the past year in order to devote more effort to investigation of the complex liver phosphodiesterases, the receptor role of ganglioside G_{M1} in choleragen activation of adenylate cyclase and the studies of cyclic GMP metabolism as outlined below. The lipase studies will be resumed as permitted by the availability of resources and the evolution of these other projects.

1. Control of Cyclic Nucleotide Phosphodiesterase and Adenylate Cyclase Activities.

After demonstrating in the past few years several ways in which cyclic nucleotide degradation can be regulated in different types of cells, we have focussed most of our attention in the past year on the phosphodiesterases of rat liver and of cultured hepatoma cells. Three cyclic GMP phosphodiesterases have been partially purified from rat liver supernatant. Some of the properties of one of these was described in last year's annual report. This phosphodiesterase appeared to be an allosteric enzyme displaying positive cooperativity between catalytic sites; cyclic GMP was preferred both as substrate and as effector (cyclic GMP > cyclic IMP > cyclic AMP). Using cyclic AMP derivatives as activators of cyclic IMP hydrolysis it was shown that the capacity to activate can be altered by modifications in the ribose, cyclic phosphate or purine (both pyrimidine and imidazole) moieties. Cyclic nucleotides also enhanced chymotryptic inactivation of the phosphodiesterase. The most potent was cyclic GMP which was active at concentrations that stimulated cyclic nucleotide hydrolysis. At similar concentrations cyclic AMP which was less effective than cyclic GMP or cyclic IMP as substrate or activator, did not enhance chymotryptic digestion. Thus interaction with the appropriate cyclic nucleotide appeared to lead to structural modification of the phosphodiesterase which was associated with increased catalytic activity and enhanced susceptibility to proteolytic attack.

The other two guanosine 3',5'-monophosphate phosphodiesterases which were partially purified from liver supernatant were not activated by cyclic nucleotides and exhibited classical kinetics. One of these enzymes was activated 8 fold by chymotrypsin. The effect appeared to be primarily on V_{max} as the Michaelis constants for cyclic GMP and cyclic AMP were unaltered. The rate

of chymotryptic activation was not influenced by cyclic nucleotides. At low substrate concentrations, this phosphodiesterase was activated by a heat- and acid-stable, protease-sensitive, calcium-dependent factor from liver supernatant. After chymotrypsin treatment, the phosphodiesterase was not further activated by the partially purified supernatant factor. The second phosphodiesterase showed significantly less activation by chymotrypsin or by the heat-stable supernatant factor. This phosphodiesterase exhibited kinetic and chromatographic properties very similar to those of the other chymotrypsin-stimulated phosphodiesterase. The sensitivities of the two enzymes to chymotrypsin stimulation were, however, quite variable and the relationship between these two enzymes remains to be established. The heat-stable phosphodiesterase activator has been purified to near homogeneity and the nature of its interactions with calcium and with the phosphodiesterases are being investigated.

We reported several years ago that cyclic AMP degradation was decreased in hepatoma cells that had been incubated with dexamethasone for >24 hr. We have now further investigated this effect which could account for the so-called permissive action of corticosteroids on cyclic nucleotide mediated processes and found after chromatographic separation of the hepatoma cell phosphodiesterases that the enzymes primarily affected by dexamethasone were cyclic GMP phosphodiesterases. Analogous effects on the liver cyclic GMP phosphodiesterases were observed after treatment of rats with dexamethasone. Applying the methods used for the purification and characterization of the liver phosphodiesterases mentioned above we are attempting to define the mechanism of this corticosteroid effect.

Adenylate cyclase studies this year have been concentrated on the mechanism of action of choleragen and evaluation of the role of ganglioside G_{M1} as the putative cell surface receptor for choleragen. Transformed mouse fibroblasts, when grown in chemically defined medium, contained no detectable G_{M1} and did not accumulate cyclic AMP in response to choleragen. Responsiveness to choleragen was demonstrated with binding of as few as 17,000 molecules of $[^3H]G_{M1}$ per cell; a maximal response was observed with 10^5 molecules per cell. With increasing cellular content of G_{M1}, the rate of rise in intracellular cyclic AMP in response to choleragen was increased. With greater than 10^5 molecules of G_{M1} per cell, the delay between addition of choleragen and the cyclic AMP response was inversely proportional to choleragen concentration. Although the cells responded to choleragen following the uptake of G_{M1} from the medium, they remained unresponsive following uptake of gangliosides G_{M2} (ca. 6×10^6 molecules per cell) and G_{M3} (ca. 2×10^6 molecules per cell). After binding G_{D1a} the cells exhibited choleragen responsiveness which could be attributed to the demonstrated enzymatic conversion of G_{D1a} to G_{M1}.

^{125}I-Choleragen binding was increased dramatically following uptake of G_{M1}, but not G_{M2} or G_{M3}, by the cells. Choleragen binding to cells containing bound exogenous G_{M1} protected the terminal galactose and the penultimate N-acetylgalactosamine of the G_{M1} from oxidation by galactose oxidase. Periodate oxidation of the sialic acid moiety of the G_{M1} was also reduced following choleragen binding. Normal human fibroblasts which respond dramatically to choleragen contained ca. 8×10^6 molecules of G_{M1} per cell. Not all of

this endogenous G_{M1} was accessible to choleragen or to enzymatic modification by galactose oxidase. Availability of the G_{M1} to galactose oxidase, however, could be increased by disruption of the cells. Binding of choleragen to these cells protected endogenous G_{M1} from oxidation by galactose oxidase, although it did not protect other glycolipids. As with the exogenous G_{M1} bound to transformed fibroblasts, periodate oxidation of the sialic acid of endogenous G_{M1} was reduced by prior incubation of intact human fibroblasts with choleragen.

These continuing studies should aid in defining the precise nature of the interaction between choleragen and ganglioside G_{M1} on the cell surface. This is a prerequisite for choleragen activation of adenylate cyclase in the intact cell although, as we have demonstrated for the first time this year, the ganglioside does not play an obligatory role in cell homogenates where the surface receptor can presumably be by-passed.

2. Metabolism of Cyclic GMP in Arterial Smooth Muscle, Leukocytes and Kidney.

We previously found that a variety of stimuli known to induce contraction of the human umbilical artery all caused accumulation of cyclic GMP in this tissue. In order to complete this study we studied $^{45}Ca^{++}$ uptake by subcellular fractions of the artery and investigated the possible role of cyclic nucleotides in this process. The characteristics of $^{45}Ca^{++}$ uptake by mitochondrial and microsomal fractions were defined but no evidence that cyclic nucleotide-dependent protein kinases could influence these processes was obtained.

In collaboration with the Laboratory of Clinical Investigation, NIAID, we found that incubation of human mononuclear cells with the ionophore A-23187 increased their cyclic AMP content, reduced the increment in cyclic GMP induced by serotonin, and inhibited serotonin-enhanced chemotaxis. In human polymorphonuclear leukocytes the ionophore also inhibited chemotaxis and increased cyclic AMP. In addition, it enhanced bidirectional Ca^{++} fluxes and lysosomal enzyme release. PGE_1 similarly inhibited chemotaxis, increased cyclic AMP and enhanced Ca^{++} fluxes but did not enhance enzyme release. These and our earlier observations on cyclic GMP are consistent with the view that elevation of intracellular cyclic GMP is associated with increased chemotactic responses and elevation of cyclic AMP with inhibition of these responses in human leukocytes.

Although not proven, it seems likely that serotonin and ascorbate increase the cyclic GMP content of monocytes (and other cells) by activating guanylate cyclase. In order to find a more practical source of cells in which to investigate this question, we screened several types of cells including alveolar macrophages, peritoneal macrophages, cells from spleen and thymus and several lines of cultured leukemia cells to assess their ability to accumulate cyclic GMP in response to serotonin or other stimuli. None of these appeared to provide a good source of guanylate cyclase for further study. We turned, therefore, to the guinea pig renal cortex in which the guanylate cyclase activity is high; a large fraction of it is soluble and is markedly stimulated by azide. Some characteristics of the

azide activation have been delineated. The enzyme from the particulate fraction which we have solubilized in good yield differs in several ways from the soluble guanylate cyclase. The latter has been partially purified using ammonium sulfate fractionation and gel chromatography. These preparations, which are stimulated little if at all by azide, are being used in an attempt to isolate (1) the factor(s) required for azide activation and (2) an apparent inhibitor of the soluble guanylate cyclase.

3. Release of Histamine from Mast Cells.

Histamine, which probably plays an important role in the pathogenesis of many allergic and inflammatory processes, can have profound effects on vascular and bronchial smooth muscle. A number of different stimuli can induce histamine release from rat mast cells in vitro. We have been able to classify these into at least three groups based on their requirements for Ca^{++} and/or phosphatidyl serine. Both of these were necessary in order to demonstrate release of histamine by antigen plus IgE, by dextran or concanavalin A. Release by protamine, polylysine, 48/80 or somatostatin was influenced little if at all by either. The ionophore A-23187 which is believed to promote the passive transfer of Ca^{++} across biological membranes required extracellular Ca^{++} for induction of histamine release but its effects were only slightly dependent on phosphatidyl serine. The action of the ionophore also differed from those of the other agents in that (1) its effects were increased in neuraminidase-treated cells whereas those of all others were decreased and (2) although most releasing agents caused "cross desensitization" to other agents, A-23187 usually produced greater rather than lesser release from cells previously exposed to low concentrations of dextran or antigen. Thus the ionophore A-23187 on the one hand and the basic agents (protamine, polylysine, 48/80) on the other apparently acted through different receptors or were able to bypass certain steps of the mechanism(s) through which antigen plus IgE, dextran and concanavalin A bring about histamine release.

It has been suggested by other workers that concanavalin A functions as a universal antigen, i.e., by combining with carbohydrate moieties of IgE molecules on the cell surface it creates bridges between them and thereby induces histamine release. If this were true the ability of a mast cell to release histamine in response to concanavalin A should be a function of the amount of IgE on its surface. We compared histamine release from mast cells of three groups of Sprague Dawley rats that might be expected to have different amounts of IgE on their surfaces. It was found rather surprisingly that mast cells from conventional, germ-free and reaginic (injected with ovalbumin and pertussis vaccine) rats all released similar amounts of histamine in response to concanavalin A. An antibody to IgE (purity uncertain) also induced release of a similar degree from cells of all three groups. Although these observations would be consistent with the conclusion that the rats of all groups had similar amounts of IgE antibody on the surfaces of their mast cells, this seems unlikely and other explanations are being sought.

There is evidence that some reactions to drugs (e.g., to acetylsalicylic acid) and other substances, although they resemble anaphylaxis, may be determined by hereditary traits and not by immunological mechanisms. Last year we reported the failure to demonstrate skin- or mast cell-sensitizing (IgE) antibodies in the serum of a strain of rats that exhibit an anaphylactoid reaction to dextran. It was possible, however, that antibodies were present on the cells of such rats even if virtually absent from the serum. Using procedures capable of transferring reactivity to ovalbumin from mast cells of rats immunized against this antigen to skin or mast cells of naive rats we were unable to transfer reactivity to dextran. These findings are consistent with all of our earlier studies which have led to the conclusion that the dextran reaction is due to the genetically determined existence of natural dextran receptors on mast cells (and perhaps other cells) and not to the presence of cytotropic antibodies. In addition, although the mechanism of the anaphylactoid reaction to dextran remains obscure, it clearly differs from that of anaphylaxis.

4. Regulation of Cholesterol Synthesis in Mammalian Cells.

We had previously shown that fibroblasts from several patients with Type II hyperlipoproteinemia exhibited a defect in feedback regulation of cholesterol synthesis and of the activity of hydroxymethylglutaryl coenzyme A reductase, the enzyme that catalyzes the rate-limiting step in cholesterol synthesis. The behavior of the cells from the NIH patients differed from that of cells from other patients studied by Goldstein and Brown and we suggested that the syndrome phenotypically designated as Type II represents more than one genotype. This view is now generally accepted. Goldstein and Brown have reported that the metabolic abnormalities in fibroblasts from patients with familial hypercholesterolemia are secondary to abnormalities in the cell surface receptors for low density lipoproteins (LDL) on these cells. We have confirmed that the binding of radiolabeled LDL at 37° is less in Type II cells than it is in normal cells. We have also shown that the binding of very low density lipoprotein is similarly depressed in Type II cells but the binding of high density lipoprotein is apparently normal. Despite differences in the magnitude of binding, however, there were no differences in the concentration or time dependency of LDL binding which might have been expected if the Type II defect involved exclusively or preferentially high affinity binding sites as postulated by other workers.

At 37° the cells not only bind LDL but also internalize it through some type of endocytic process. At 4° binding occurs but endocytosis is minimal. We found as have others that no significant impairment of LDL binding by Type II cells was demonstrable at 4° over a wide range of LDL concentrations. When cells that had bound LDL at 4° were repeatedly washed the elution of LDL from normal and Type II cells did not differ appreciably. Removal of LDL bound to fibroblasts by incubation of the cells with trypsin likewise followed a similar course in the normal and Type II cells. It would appear that the latter cells may have a defect in the mechanism(s) for internalization of LDL rather than in the binding per se. In collaboration with the Section on Molecular Pharmacology, Pulmonary Branch, NHLI, the microviscosities of cell membranes of normal and Type II fibroblasts were compared using electron spin

resonance and fluorescent polarization techniques. No alterations in the fluidity of the plasma membranes of the Type II cells were demonstrable.

In other studies the relationship between cholesterol synthesis and cell proliferation was investigated. In addition the effects of insulin and protein synthesis inhibitors on the turnover of the cell surface binding sites for LDL have been described and effects of LDL itself on the concentration of receptor sites were demonstrated. It has been reported that pure cholesterol did not depress cholesterol synthesis in mouse fibroblasts whereas products of sterol oxidation were markedly inhibitory. We undertook, thereafter, to determine whether oxidation of cholesterol in serum lipoproteins during the course of their preparation might contribute to their effects and have concluded that oxidized sterols probably do not play a significant part in the inhibition of cholesterol synthesis produced by lipoproteins in vitro. We have recently found that brief incubation of human skin fibroblasts with digitonin causes marked stimulation of cholesterol synthesis which continues to increase for several hours. Digitonin presumably interacts relatively specifically with cholesterol in the plasma membrane and may serve as a very useful probe for evaluation of the role of this component in regulation of cholesterol synthesis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOL HL 00601-07 CM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Sterol Synthesis in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Avigan Research Chemist CM NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.8	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied factors influencing specific binding of low density serum lipoproteins (LDL) to normal and type II homozygous hyperlipoproteinemia skin fibroblasts as well as conditions affecting sterol synthesis in human and animal fibroblast cells in culture. Binding requires protein synthesis since it is reduced by cycloheximide. Binding is also subject to certain hormonal control. Type II condition is not simply a deficiency in binding but it affects LDL intracellular transport. The correlation between cellular proliferation and the role of lipid synthesis has been investigated. The effect of factors stimulating intracellular concentration of cyclic AMP on sterol synthesis has also been studied.

PHS-6040
(12-75)

Objectives and background

Human skin fibroblasts bind, internalize and metabolize low density serum lipoproteins (LDL). This interaction activates a number of regulatory mechanisms, in which the LDL, or the cholesterol contained therein, affects reactions leading to sterol synthesis and metabolism. The nature and specificity of binding of serum lipoproteins by normal and type II hyperlipoproteinemic human fibroblasts have been investigated. Studies on the control of sterol synthesis as well as the influence of various factors on this process have been continued.

Methods

Confluent cell cultures of human skin fibroblasts and of mouse 929 cells were used. Cells were incubated, usually overnight, in various media and washed with buffer. For LDL binding experiments they were exposed to ^{125}I-labeled LDL under controlled conditions. Excess label was removed by a standardized washing procedure and the residual bound radioactivity assayed. For experiments on sterol synthesis cells were incubated with labeled acetate, the nonsaponifiable lipids were isolated and their radioactivity determined.

Findings

I. Various factors affecting LDL binding to fibroblasts.

1) When protein synthesis was inhibited by cycloheximide, binding measured at 4° as well as binding and endocytosis followed at 37° decreased with a half-time of about 12 hr. On removal of cycloheximide, binding capacity began to increase and was fully restored after 24 hr.

2) When cells were incubated at 37° with native LDL, the binding of ^{125}I-LDL declined with a half-time of about 10 hr. Because of its relative slowness the decline very likely resulted from the reduction in the concentration of receptor sites rather than from their becoming occupied by the native LDL. This would seem to indicate the feedback regulation of LDL binding.

3) Contrary to reports by others, it was found that insulin increased the binding of LDL by fibroblasts. Binding did not acutely depend on the presence of free sulhydryl groups since it was not suppressed by short-term incubations of cells with NEM or PCMB.

II. Studies on the nature of the specific interaction of LDL with fibroblasts.

Numerous attempts by a number of methods were made to demonstrate high affinity specific binding of LDL.

1) Although it was repeatedly confirmed that the type II homozygous cells have a significantly lower ability than normal cells to interact at 37° with labeled LDL at a wide range of concentrations, there was no convincing evidence for a basic difference in the concentration or time dependency of the interactions. Such differences could have been expected if the type II deficiency affected exclusively or preferentially the high affinity interactions, as proposed by others.

2) When cells that had bound ^{125}I-LDL at 0° were repeatedly washed with buffer, the elution of ^{125}I from type II cells (presumably devoid of specific binding sites) and normal cells was not demonstrably different. The removal of ^{125}I-LDL bound to fibroblasts by digestion with trypsin also followed a similar course in normal and type II cells.

3) Other workers had found that although type II fibroblasts showed defective interaction with LDL at 37° (when considerable endocytosis is taking place), there was no significant impairment at 4°. We confirmed this finding over a wide range of LDL concentrations with cells from one type II patient, suggesting that the abnormality depends on deficiency in the transport mechanism rather than in the binding per se. No specific deficiency of high affinity binding of LDL by type II cells was demonstrated.

III. Binding of VLDL and HDL by fibroblasts.

^{125}I-VLDL binding was approximately proportional to concentration over a wide range and was not demonstrably saturable. Homozygous type II cells bound appreciably less VLDL than did normal cells at a given concentration. On the other hand, there was no deficiency in binding or uptake of ^{125}I-HDL by the type II cells. LDL at high concentration did not appreciably interfere with HDL binding in either normal or type II cells.

IV. Correlation between cellular proliferation and the rate of lipid synthesis.

Since growing cells require a supply of cholesterol to build new membranous structures, one might expect that conditions favoring cellular proliferation might also stimulate cholesterol synthesis. We have looked for such a correlation by following the incorporation of labeled acetate into cholesterol and of thymidine into DNA in fibroblasts. It was shown that the effect on sterol synthesis did not strictly parallel the effect on DNA synthesis, since in confluent skin fibroblast cultures insulin caused only a minimal or no increase in DNA synthesis, while it increased sterol synthesis, usually several fold. Similarly, the effect of LDL was also quite specific and limited to the inhibition of cholesterol synthesis with little or no change in DNA synthesis.

V. Correlation between the mechanisms for removal of glucocorticoids from cells and their metabolic effects in diploid and transformed fibroblasts.

As previously reported (NHLI-276, 1973), dexamethasone stimulates lipid synthesis in several diploid cell lines, but not in transformed ones. In experiments in which [^{3}H]-dexamethasone was incubated with cells, we confirmed findings by Gross, et al. (J. Cell Biol. 44: 103, 1970) on the active process for removal of glucocorticoids from L-929 mouse fibroblasts. No similar mechanism, however, was demonstrated in human skin fibroblasts.

VI. The effect of intracellular concentration of cyclic AMP on sterol synthesis.

It was reported by Beg et al. (Biophys. Biochem. Res. Comm. 54: B62, 1973) that ATP and Mg inhibit the activity of HMGCoA reductase in liver preparations and that cyclic AMP augments this effect. It was also shown by V. C. Manganiello and J. Breslow (Biochim. Biophys. Acta 362: 509, 1974) that prostaglandin E_1 (PGE_1) rapidly elevates cyclic AMP in skin fibroblasts. We looked for an acute effect of PGE_1 on the rate of sterol synthesis which normally is limited by the activity of HMGCoA reductase in these cells. The results were negative and no direct effect of PGE_1 and therefore of elevated cyclic AMP on sterol synthesis could be demonstrated.

VII. Stimulation of sterol synthesis by digitonin.

It was found that a short-term incubation of human skin fibroblasts with digitonin (2-25 µg/ml for 10-60 min) caused marked enhancement of the rate of cholesterol synthesis which continued to increase for a few hours after removal of digitonin. The effect of insulin on sterol synthesis was unchanged in digitonin-treated cells, although the latter abolishes certain other metabolic effects of the hormone (R. A. Akhtar and M. C. Perry, Biochim. Biophys. Acta 411: 30, 1975). Stimulation of sterol synthesis by digitonin is likely a result of a specific interaction with plasma membrane cholesterol and may provide information on the role of this component in the control of cholesterol synthesis.

VIII. The effect of antioxidants.

It was reported that suspensions of pure cholesterol did not inhibit cholesterol synthesis in mouse fibroblasts in culture, while oxidized sterol derivatives were strongly inhibitory (A. A. Kandutsch and H. W. Chen, J. Biol. Chem. 249: 6057, 1974). We attempted to determine whether native serum lipoproteins in which autoxidation had been prevented might also fail to inhibit cholesterol synthesis. Human blood was withdrawn and serum was prepared anaerobically in the presence of α-tocopherol. When such serum was incubated with human skin or mouse fibroblasts, the inhibitory effects were not substantially different from those obtained with conventionally prepared serum; nor was it possible to reverse serum inhibition of cholesterol

synthesis by adding to the incubation chemical antioxidants (BHT, DTT). These results suggest that, barring the presence of oxidized sterols in native human lipoproteins, the inhibition of sterol synthesis in vitro is caused by lipoproteins containing unoxidized cholesterol.

Significance to Heart and Lung Research:

Studies on the control of sterol synthesis in mammalian cells grown in culture are useful for the understanding of problems related to the metabolism of serum lipoproteins and of cholesterol in the human organism. Research on the nature of the type II abnormality could eventually lead to a better understanding of the connection between certain metabolic defects and atherosclerosis.

Proposed Course:

It is proposed to investigate further hormonal effects on sterol synthesis in fibroblasts and in other cell lines. It is also proposed to study processes reducing the concentration of cellular free cholesterol through esterification, as well as release in the free and complex form.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00602-06 CM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Histamine Release from Mast Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. H. Baxter Medical Officer CM NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

SUMMARY OF WORK (200 words or less - underline keywords)

The object is to elucidate the cellular mechanisms involved in histamine release from mast cells. The present studies are concerned with: (1) the basis of anaphylactoid reactivity, and (2) differences in requirements and actions of different histamine releasing agents.

PHS-6040

Project Description:

Basis of anaphylactoid reactivity. There is evidence that some reactions to drugs such as acetylsalicylic acid (ASA) and other substances, although resembling anaphylaxis, may be determined by hereditary traits and not by immunological mechanisms. Last year we reported the failure to find skin- and mast cell-sensitizing (IgE) antibodies in serum of a strain of rats that react to dextran. Because of the possibility that antibodies might be present on the mast cells of such rats, even if virtually absent from the serum, we have now eluted the mast cells with pH 3 buffer and examined the eluted material. The material consistently failed to transfer anti-dextran reactivity to the skin or mast cells of non-dextran-reactive rats. When the dextran-reactive donor rats had also been immunized with egg albumin (EA) and pertussis vaccine (which induced IgE antibodies against EA), the eluted material readily transferred reactivity against EA. This indicated that the technical procedures were adequate to identify anti-dextran IgE antibodies if they had been present.

Requirements and actions of different histamine-releasing agents. To determine whether the release reaction induced in rat mast cells by different agents is the same, we compared a number of agents. The agents could be divided into several groups, based on differences in their requirement for Ca^{++} and phosphatidyl serine (PS) in the medium. Dextran and concanavalin A (con A), and (with IgE-sensitized cells) specific antigen and anti-IgE, required Ca^{++} and PS. A-23187 required Ca^{++} but was only slightly dependent on PS. Protamine, polylysine, 48/80 and probably somatostatin had little or no requirement for either Ca^{++} or PS. Pretreatment of the cells with neuraminidase (2 experiments) decreased release (in normal buffer) by all agents except A-23187; release by A-23187 was increased. Exposure of cells to a low concentration of any one of the releasing agents desensitized the cells against higher concentrations of the same agent. In addition, most of the agents caused cross-desensitization against other agents. The most notable exception was the failure of dextran or antigen to desensitize against A-23187; A-23187 usually produced greater rather than lesser release after the cells had been exposed to low concentrations of dextran or antigen. Thus, some agents (notably A-23187 and the basic agents) differed from other agents in their requirements for release, or in ability to cross-desensitize. Possibly they acted on different receptors, or were able to bypass certain steps of the release mechanism.

It has been suggested that con A acts as a universal antigen - combining with carbohydrate of the IgE antibody on mast cells, bridging the molecules, and thus releasing histamine. We determined histamine release by con A from mast cells of three groups of Sprague-Dawley rats: conventional, germ-free and reaginic (i.e., injected with EA and pertussis vaccine). Significant release of similar degree was obtained from all three groups. This suggested, based on the proposal outlined above, that rats of all groups had similar amounts of IgE antibody on the mast cells, which appeared unlikely. However, anti-IgE antibody (of uncertain purity) also induced release of similar degree from cells of all groups. (The result is puzzling.)

We plan to complete the studies described here. In addition, we shall study dextran-binding by mast cells (and other cells), which will necessitate further work on methods for obtaining purified mast cells that retain the capacity to release histamine in response to the several stimuli mentioned above.

Significance to Heart and Lung Research:

Histamine and other mediators released from mast cells produce important effects on small blood vessels and bronchial smooth muscle.

Publications:

Baxter, J. H., and Adamik, R. Effects of calcium and phosphatidyl serine in rat mast cell reaction to dextran. Proc. Soc. Exp. Biol. Med. in press.

Baxter, J. H., and Adamik, R. Studies on passive transfer of anti-dextran and anti-egg albumin reactivity in the rat. Int. Arch. Allergy, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00603-02 CM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Rat Liver Phosphodiesterases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Moss	Research Associate	CM NHLI
OTHER:	M. Vaughan	Chief, Lab. Cellular Metab.	CM NHLI
	V. C. Manganiello	Medical Officer (Research)	CM NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of the project are to determine the factors responsible for regulation of intracellular cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) content. The phosphodiesterases responsible for cyclic nucleotide hydrolysis were partially purified from rat liver. Three guanosine 3',5'-monophosphate phosphodiesterases were identified, partially purified, and their regulatory, structural and kinetic properties determined.

PHS-6040
(12-75)

Project Description:

Objectives. To define the regulatory, structural and kinetic properties of the phosphodiesterases responsible for cyclic nucleotide hydrolysis in rat liver.

Methods. Phosphodiesterase activity was measured by methods previously described in this laboratory. The enzymes were purified by ultracentrifugation and affinity chromatography.

Major Findings. Three guanosine 3',5'-monophosphate phosphodiesterases were partially purified from rat liver supernatant. The properties of one of the phosphodiesterases was described in last year's annual report. This phosphodiesterase appeared to be an allosteric enzyme displaying positive cooperativity between catalytic sites; cyclic GMP was preferred as both substrate and effector (cGMP > cIMP > cAMP). Using cAMP derivatives as activators of cIMP hydrolysis it was shown that the capacity to activate is altered by modifications in the ribose, cyclic phosphate or purine (both pyrimidine and imidazole) moieties. Cyclic nucleotides also enhanced chymotryptic inactivation of the phosphodiesterase. The most potent was cGMP which was active at concentrations that stimulated cyclic nucleotide hydrolysis. At similar concentrations cAMP which was less effective than cGMP or cIMP as substrate or activator, did not enhance chymotryptic digestion. On interaction with the appropriate cyclic nucleotide this phosphodiesterase appeared to undergo structural modification which was associated with increased catalytic activity and enhanced susceptibility to proteolytic attack.

The other two guanosine 3',5'-monophosphate phosphodiesterases which were partially purified from rat liver supernatant were not activated by cyclic nucleotides and exhibited classical kinetics. One phosphodiesterase was activated 8-fold by chymotrypsin. The effect appeared to be primarily on V_{max} as the K_m's for cGMP (4 μM) and cAMP (50 μM) were unaltered. The rate of chymotryptic activation was not affected by cyclic nucleotides. At low substrate concentration (0.3 μM) this phosphodiesterase was activated by a heat- and acid-stable, chymotrypsin-sensitive, calcium-dependent factor from liver supernatant. After chymotrypsin treatment, the phosphodiesterase was not further activated by the partially purified supernatant factor. The second phosphodiesterase showed significantly less activation by chymotrypsin or by the heat-stable supernatant factor. This phosphodiesterase exhibited similar kinetic and chromatographic properties to the other chymotrypsin-stimulated phosphodiesterase. The sensitivities of the two enzymes to chymotrypsin stimulation were, however, quite variable and perhaps related to the physiological status of the animals from which the livers were obtained.

Significance to Heart and Lung Research:

Control over intracellular cAMP and cGMP levels may be important for the regulation of smooth muscle contraction in both the pulmonary and cardiovascular systems.

Proposed Course:

The phosphodiesterases will be further purified and their regulatory properties investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00604-02 CM

PERIOD COVERED July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of Salicylates on Histamine Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Moss	Research Associate	CM NHLI
	Maria C. de Mello	Visiting Fellow	PB NHLI
Others:	M. Vaughan	Chief, Lab. Cellular Metab.	CM NHLI
	M. A. Beaven	Research Pharmacologist	PB NHLI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLI

Dr. de Mello is supported by the Brazilian National Research Council.

LAB/BRANCH Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

In man and other laboratory animals, urinary excretion of the histamine metabolite, imidazoleacetate, is increased and that of its conjugated metabolite, ribosylimidazoleacetate, decreased by salicylates. Since imidazoleacetate has been reported to produce analgesia and narcosis, its accumulation as a result of transferase inhibition could play a part in the therapeutic effects of salicylates. In order to determine the locus of salicylate action, we investigated the effect of anti-inflammatory drugs on imidazoleacetate phosphoribosyl transferase, the enzyme that catalyzes the ATP-dependent conjugation of imidazoleacetate with ribose-5-phosphate. As little as 0.2 mM aspirin produced 50% inhibition of the rat liver transferase. In vivo, a 30% decrease in the urinary excretion of ribosylimidazoleacetate has been observed with plasma salicylate concentrations of 0.4 mM. The enzyme was also inhibited by sodium salicylate but not by salicylamide, sodium gentisate, aminopyrine, phenacetin, phenylbutazone, or indomethacin. The last four drugs have been shown not to alter the excretion of ribosylimidazoleacetate when administered in vivo. Since both the drug specificity and inhibitory concentrations are similar in vivo and in vitro, it seems probable that the effect of salicylates on imidazoleacetate conjugation results from inhibition of imidazoleacetate phosphoribosyl transferase.

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PHS-6040
(12-75)

Project Description:

Objectives: To define the mechanism and specificity for the *in vivo* inhibition by salicylates of the formation of the histamine metabolite, 5'-phosphoribosylimidazoleacetate.

Methods: Imidazoleacetate phosphoribosyl transferase was isolated by standard chromatographic and ultracentrifugal procedures. Product identification was based on methods developed by Beaven et al. (European J. Pharm. 29: 138-146, 1974).

Major Findings: Beaven and coworkers previously demonstrated that the administration of sodium salicylate or aspirin alters the metabolism of histamine in several animal species. The formation of the histamine metabolite, 5'-phosphoribosylimidazoleacetate, was inhibited by these salicylates, but not by other anti-inflammatory agents. In order to determine the locus of salicylate action, the enzyme responsible for the conversion of imidazoleacetate to its phosphoribosyl derivative was purified from rat liver. As little as 0.2 mM aspirin produced 50% inhibition of the imidazoleacetate phosphoribosyl transferase. *In vivo*, a 30% decrease in the urinary excretion of ribosylimidazoleacetate has been observed with plasma salicylate concentrations of 0.4 mM. The enzyme was also inhibited by sodium salicylate, but not by salicylamide, sodium gentisate, aminopyrine, phenacetin, phenylbutazone, or indomethacin. The last four drugs do not alter the excretion of ribosylimidazoleacetate when administered *in vivo*. Since both the drug specificity and inhibitory concentrations are similar *in vivo* and *in vitro*, it seems probable that the effect of salicylates on imidazoleacetate conjugation results from inhibition of imidazoleacetate phosphoribosyl transferase.

Significance to Heart and Lung Research:

Histamine exerts a profound effect on the smooth muscle of the lung. Inhibition of one of the pathways for histamine degradation by salicylates may thus be relevant in the pathogenesis of certain disease states.

Proposed Course:

No further investigation in this area is anticipated.

Publications:

Moss, J., de Mello, M. D., Vaughan, M., and Beaven, M. S. Effect of salicylates on histamine and L-histidine metabolism. Inhibition of imidazoleacetate phosphoribosyl transferase. J. Clin. Invest., 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00605-03 CM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide Metabolism in Human Umbilical Artery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. C. Manganiello	Medical Officer (Research)	CM NHLI
	R. I. Clyman	Staff Associate	CM NHLI
Other:	M. Vaughan	Chief, Lab. Cellular Metabolism	CM NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

SUMMARY OF WORK (200 words or less - underline keywords)

Ca^{++} uptake by subcellular fractions of human umbilical artery was investigated. No effects of cyclic nucleotides were demonstrable.

This project has been terminated.

PHS-6040
(12-75)

Objectives:

To determine the effects of divalent cations and oxygen on cyclic nucleotide metabolism in the human umbilical artery, and to describe the systems of Ca^{++} uptake in this tissue.

Methods:

Umbilical cords from term (gestational) pregnancies were obtained within 30 min of delivery. Arteries were incubated or homogenized and subcellular fractions obtained for measurement of Ca^{++} uptake by standard methods.

Major Findings:

Two different mechanisms for the active accumulation of Ca^{++} by subcellular fractions of human umbilical artery are described. One, located in the mitochondrial fraction, was induced by exogenous ATP or respiratory substrates (ADP and succinate) and was inhibited by azide. The other, located in the microsomal fraction, was induced by ATP and potentiated by oxalate but not inhibited by azide. Increasing ATP concentrations up to 4 to 5 mM increased microsomal Ca^{++} accumulation whereas increasing ATP concentration above 2 to 3 mM caused inhibition of mitochondrial Ca^{++} uptake. Although changing pH from 7.4 to 7.2 had no effect on mitochondrial Ca^{++} accumulation, it doubled microsomal uptake. Neither adenosine 3',5'-monophosphate nor guanosine 3',5'-monophosphate in the presence or absence of protein kinase and kinase modulator affected Ca^{++} uptake by or phosphorylation of the subcellular fractions. Partially purified protein kinases from umbilical and beef skeletal muscle contained a component(s) distinguishable from the kinase on the basis of its heat stability that enhanced ATP induced Ca^{++} uptake by mitochondrial fractions from the umbilical artery. It is suggested that alterations in Ca^{++} sequestration induced by changes in ATP concentration and intracellular pH in mitochondrial and microsomal fractions respectively could play a role in the control of arterial patency and closure with changes in pO_2.

This project has been terminated.

Publications:

Clyman, R. I., Sandler, J. A., Manganiello, V. C., and Vaughan, M. Guanosine 3',5'-monophosphate and adenosine 3',5'-monophosphate content of human umbilical artery. J. Clin. Invest. 55: 1020-1025, 1975. (Correction of error in 1975 report.)

Clyman, R. I., Blacksin, A. S., Sandler, J. A., Manganiello, V. C., and Vaughan, M. Role of Ca^{++} in the regulation of cyclic nucleotide content in human umbilical artery. J. Biol. Chem. 250: 4718-4721, 1975.

Clyman, R. I., Blacksin, A. S., Manganiello, V. C., and Vaughan, M. Oxygen and cyclic nucleotides in human umbilical artery. Proc. Nat. Acad. Sci. USA 72: 3883-3887, 1975.

Clyman, R. I., Manganiello, V. C., Lovell-Smith, C. J., and Vaughan, M. Calcium uptake by subcellular fractions of human umbilical artery. Am. J. Physiol., September 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00606-05 CM

PERIOD COVERED July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide Metabolism in Cultured Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Moss	Research Associate	CM NHLI
OTHER:	V. C. Manganiello	Medical Officer (Research)	CM NHLI
	M. Vaughan	Chief, Lab. Cellular Metab.	CM NHLI
	P. H. Fishman	Research Biochemist	DMNB NINCDS

COOPERATING UNITS (if any)

Developmental and Metabolic Neurology Br., NINCDS

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	0.9	1.1

SUMMARY OF WORK (200 words or less - underline keywords)

Transformed mouse fibroblasts, when grown in chemically defined medium, contained no detectable G_{M1} and did not accumulate cyclic AMP in response to choleragen. Responsiveness to choleragen was observed following the binding of the monosialoganglioside G_{M1}. ^{125}I-Choleragen binding was increased dramatically following uptake of G_{M1}, but not G_{M2} or G_{M3} by the cells. Although uptake of G_{M1} was essential for in vivo responsiveness to choleragen, the in vitro activation of adenylate cyclase by choleragen did not require integration of G_{M1} into the cells.

Choleragen binding to cells containing bound exogenous G_{M1} protected the G_{M1} from oxidation by galactose oxidase and periodate. Normal human fibroblasts containing endogenous G_{M1} responded to choleragen, choleragen binding protecting the G_{M1} from galactose oxidase and periodate. All the endogenous G_{M1} was not accessible to choleragen or to enzymatic modification by galactose oxidase. Availability of the G_{M1} to galactose oxidase, however, could be increased by disruption of the cells.

PHS-6040
(12-75)

Project Description:

Objectives. To study control of cyclic AMP and cyclic GMP metabolism in cultured cells. In the past year, normal and transformed fibroblasts have been used to investigate the mechanism of adenylate cyclase activation by choleragen and the role of gangliosides in the binding of choleragen to the cell surface.

Methods. Measurement of cyclic AMP by the method of Gilman (Proc. Nat. Acad. Sci. 67: 305, 1970); adenylate cyclase, phosphodiesterase, choleragen binding and labeling, by methods to be reported; ganglioside labeling, binding, synthesis and degradation by our methods published and unpublished.

Major Findings.

(1) Functional incorporation of ganglioside into intact cells: relationship of ganglioside structure to choleragen response. Transformed mouse fibroblasts, when grown in chemically defined medium, contained no detectable G_{M1} and did not accumulate cyclic AMP in response to choleragen. Responsiveness to choleragen was demonstrated with binding of as few as 17,000 molecules of $[^3H]G_{M1}$ per cell; a maximal response was observed with 10^5 molecules per cell. With increasing cellular content of G_{M1}, the rate of rise in intracellular cyclic AMP in response to choleragen was increased. With greater than 10^5 molecules of G_{M1} per cell, the delay between addition of choleragen and the cyclic AMP response was inversely proportional to choleragen concentration. Although the cells responded to choleragen following the uptake of G_{M1} from the medium, they remained unresponsive following uptake of gangliosides G_{M2} (approx. 6×10^6 molecules per cell) and G_{M3} (approx. 2×10^6 molecules per cell). A choleragen response was observed when the cells had bound approximately 2×10^7 molecules of G_{M2} per cell. After binding G_{D1a} the cells exhibited choleragen responsiveness which could be attributed to the enzymatic conversion of G_{D1a} to G_{M1}. ^{125}I-Choleragen binding was increased dramatically following uptake of G_{M1}, but not G_{M2} or G_{M3}, by the cells.

Although uptake of G_{M1} was essential for *in vivo* responsiveness to choleragen, the *in vitro* activation of adenylate cyclase by choleragen did not require integration of G_{M1} into the cells. *In vitro* activation of the cyclase was dependent on NAD, as has been noted in other cell-free systems.

Choleragen binding to cells containing bound exogenous G_{M1} protected the terminal galactose and the penultimate N-acetylgalactosamine of the G_{M1} from oxidation by galactose oxidase. Periodate oxidation of the sialic acid moiety of the G_{M1} was also reduced following choleragen binding.

(2) Normal human fibroblasts which respond dramatically to choleragen synthesized radiolabeled G_{M1} from $[^{14}C]$galactose and $[^3H]$N-acetylmannosamine, and contained approximately 8×10^6 molecules of G_{M1} per cell. All the endogenous G_{M1} was not accessible to choleragen or to enzymatic modification by galactose oxidase. Availability of the G_{M1} to galactose oxidase, however, could be increased by disruption of the cells. Binding of choleragen to

these cells protected endogenous G_{M1} from oxidation by galactose oxidase, although it did not protect other glycolipids. As with the exogenous G_{M1} of NCTC 2071 cells, periodate oxidation of the sialic acid of endogenous G_{M1} was reduced by prior incubation of intact cells with choleragen.

Significance to Heart and Lung Research:

Cultured cells which can be grown and studied in a controlled environment (in some instances in a completely chemically defined medium) are particularly useful for defining the mechanisms by which hormones and other agents (e.g., cholera toxin) act to control cyclic nucleotide metabolism. Information from these systems is valuable in understanding the effects of hormones, prostaglandins and other humoral agents on the pulmonary and cardiovascular systems.

Proposed Course:

The interaction of choleragen with surface receptors will be investigated in normal and transformed fibroblasts following chemical and enzymatic modification of the cell surface, as well as in model systems.

Publications:

Moss, J., Fishman, P. H., Manganiello, V. C., Vaughan, M., and Brady, R. O. Functional incorporation of ganglioside into intact cells: induction of choleragen responsiveness. Proc. Nat. Acad. Sci. USA 73: 1034, 1976.

Fishman, P. H., Moss, J., and Vaughan, M. Uptake and metabolism of gangliosides in transformed mouse fibroblasts: relationship of ganglioside structure to choleragen response. J. Biol. Chem, 1976, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00607-03 CM

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cyclic GMP Metabolism in Leukocytes, Lung and Kidney

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.-C. Tsai	Research Chemist	CM NHLI
OTHER:	M. Vaughan	Chief, Laboratory of Cellular Metabolism	CM NHLI
	V. C. Manganiello	Medical Officer (Research)	CM NHLI
	J. I. Gallin	Senior Investigator	LCI NIAID

COOPERATING UNITS (if any)

Laboratory of Clinical Investigation, NIAID

LAB/BRANCH
Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

SUMMARY OF WORK (200 words or less - underline keywords)

In studies of the effects of serotonin, ascorbic acid, prostaglandin E_1 and ionophore A-23187 on human leukocytes, it was found that agonists that increase cyclic GMP enhance chemotaxis whereas those that increase cyclic AMP inhibit it. Several lines of cultured lymphocytic leukemia cells have been characterized in terms of cyclic GMP content, responsiveness to serotonin and guanylate cyclase activity. Soluble and solubilized particulate guanylate cyclases from renal cortex have been partially purified and compared.

PHS-6040
(12-75)

Project Description:

Objectives. (1) To establish relationships between cyclic GMP metabolism and function in leukocytes, lung and kidney, and (2) to determine the mechanisms through which guanylate cyclase activity is regulated.

Methods. Cells or tissue slices were prepared and incubated, cyclic nucleotides were extracted, purified and quantified by standard methods. Chemotaxis was evaluated by a modification of the Boyden chamber procedure. Guanylate cyclase was assayed by radioimmunoassay of the cyclic GMP formed from GTP.

Major Findings.

(1) We reported last year that serotonin, acetylcholine and ascorbate increase the cyclic GMP content of human mononuclear cells and enhance chemotaxis. The ionophore A-23187, which increases cyclic GMP in umbilical artery segments, did not increase the cyclic GMP content of human mononuclear cells but rather increased their cyclic AMP content, reduced the increment in cyclic GMP induced by serotonin, and inhibited serotonin-enhanced chemotaxis. In human polymorphonuclear leukocytes the ionophore also inhibited chemotaxis and increased cyclic AMP. In addition, it enhanced bidirectional Ca^{++} fluxes and lysosomal enzyme release. PGE_1 similarly inhibited chemotaxis, increased cyclic AMP and enhanced Ca^{++} fluxes but did not enhance enzyme release. These and our earlier observations are consistent with the view that elevation of intracellular cyclic GMP is associated with increased chemotactic responses and elevation of cyclic AMP with inhibition of these responses in human leukocytes. (These studies were carried out in collaboration with Dr. John Gallin, NIAID.)

(2) Although not proven, it seems likely that serotonin and ascorbate increase the cyclic GMP content of monocytes or circulating macrophages (and other cells) by activating guanylate cyclase. In order to find a more practical source of cells in which to investigate this question, we screened several types of cells to assess their ability to accumulate cyclic GMP in response to serotonin or other stimuli. Rabbit alveolar macrophages (from pulmonary lavage) were unresponsive to serotonin, ascorbate and several other potential agonists. (The cyclic AMP content of the alveolar macrophages was increased about 50% by PGE_1). Guinea pig peritoneal macrophages elicited with several different stimuli also failed to respond to serotonin or ascorbate with a rise in cyclic GMP. In cells from guinea pig spleen the cyclic GMP content was increased 10 fold by serotonin or ascorbate. Cells from rat spleen, however, failed to respond to these agonists although cells from rat thymus did accumulate cyclic GMP on incubation with serotonin or azide.

Because the preparations from spleen or thymus were heterogeneous and variable in cellular composition, we investigated several lines of human lymphocytic leukemia cells that are maintained in culture. In several lines of T cells serotonin increased cyclic GMP levels to a variable extent. In

one line (8402) the responses to serotonin were particularly large, about 8-fold. The cyclic AMP content of these cells was not altered by serotonin. (It was increased several fold by PGE_1.) These cells also had relatively high levels of guanylate cyclase activity, a large fraction of which was soluble and was stimulated by azide. For reasons that were not clear, however, the responsiveness of these cells and their guanylate cyclase activity varied greatly in different subcultures. In two lines of B lymphocytes serotonin was without effect on cyclic GMP content. The guanylate cyclase in these cells was lower than that of the T cells studied and was not stimulated by azide.

None of the cultured cells screened appeared to provide a good source of guanylate cyclase for further study. We turned, therefore, to the guinea pig renal cortex in which the guanylate cyclase activity is high; a large fraction of it is soluble and is markedly stimulated by azide. After ammonium sulfate fractionation, total basal activity was markedly increased but the percentage stimulation by azide was greatly decreased. Some characteristics of the azide activation have been delineated. The ammonium sulfate precipitated enzyme has been further purified using gel chromatography and these preparations, which are stimulated little if at all by azide, are being used in an attempt to isolate (1) the factor(s) required for azide activation and (2) an apparent inhibitor of the soluble guanylate cyclase. The enzyme from the particulate fraction which we have solubilized in good yield differs in several ways from the soluble guanylate cyclase.

(3) Several years ago we reported findings consistent with the view that acetylcholine and bradykinin increase prostaglandin synthesis in lung as a consequence of their effects on cyclic GMP metabolism. We have now found evidence that bradykinin has analogous effects in the guinea pig kidney medulla but not in the cortex.

Significance to Heart and Lung Research:

Although at present information concerning the metabolism and functions of cyclic GMP is limited it appears that this nucleotide may be of especial importance in the development, physiology and pathology of lung, vascular smooth muscle and kidney.

Proposed Course:

We shall further purify the guanylate cyclases from kidney (and perhaps from lung) in order to elucidate the mechanisms by which their activity is controlled.

Publications:

Sandler, J. A., Gallin, J. I., and Vaughan, M. Effects of serotonin, carbamylcholine, and ascorbic acid on leukocyte cyclic GMP and chemotaxis. J. Cell Biol. 67: 480-484, 1975.

Sandler, J. A., Smith, T. K., Manganiello, V. C., and Kirkpatrick, C. H. Stimulation of monocyte cGMP by leukocyte dialysates. J. Clin. Invest. 56: 1271-1279, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00608-03 CM

PERIOD COVERED July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Cyclic AMP Phosphodiesterase Activity in Fat Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. C. Manganiello	Medical Officer (Research)	CM NHLI
Other:	M. Vaughan	Chief, Lab. Cellular Metabolism	CM NHLI
	C. J. Lovell-Smith	Visiting Associate	CM NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.2	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

We have solubilized 80-100% of the particulate phosphodiesterase (PDE) with a combination of 30 mM Tris HCl, pH 8.0, 3.0 mM $MgSO_4$, 0.1% Brij 30, 1% Triton X-100, 5 mM DTT, 5 mM NaBr. The solubilized enzyme exhibits low Michaelis constants (ca. 0.6 μM) for cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP). The increased activity of the particulate PDE produced by incubation of fat cells with insulin or ACTH is maintained in the solubilized state. The solubilized enzyme exhibits 2 peaks on PAGE electrophoresis (3.5% gels) and DEAE chromatography. cAMP and cGMP PDE activity co-elute; the eluted material exhibit low Michaelis constants for cAMP and cGMP.

184

PHS-6040
(12-75)

Project Description:

We have solubilized the particulate fat cell phosphodiesterase(s) that is subject to control by insulin, glucocorticoids, cyclic AMP and T_3, and have begun to characterize the solubilized enzyme.

Initially, the "low K_m" particulate diesterase was solubilized with a combination of the non-ionic detergent, Brij 30 (0.1%) and 1 M NaCl. NaBr was as effective as NaCl. Further studies indicated that in the presence of 5 mM DTT, Brij 30 (0.1%) and 40 mM NaBr, 80% of the activity could be solubilized. When these solutions were stored for more than 12 hr, however, the Brij 30 tended to aggregate and precipitate. To stabilize and clarify the solubilizing medium, 1% Triton X-100 was added. The final solubilization medium (30 mM Tris Cl, pH 8.0, 3 mM $MgSO_4$, 5 mM DTT, 5 mM NaBr, 0.1% Brij 30, 1% Triton X-100) solubilized 80 to 100% of P_2 particulate phosphodiesterase activity.

The solubilized enzyme exhibited low Michaelis constants for both cAMP and cGMP, and the increased phosphodiesterase activity produced by incubation of fat cells with insulin or ACTH was maintained in the solubilized preparations.

Polyacrylamide gel electrophoresis of the solubilized enzyme on 3.5% gels indicated that two peaks of activity were present; both peaks hydrolyzed cAMP and cGMP. The solubilized enzyme was bound to DEAE Biogel and could be eluted as a single asymetrical peak or a broad band of activity with one or more peaks; cAMP and cGMP hydrolyzing activity co-eluted. Attempts to further separate various forms of the solubilized diesterase by affinity chromatography or phosphocellulose chromatography were not successful. In several experiments we were unable to demonstrate that in fat cells incubated with $^{32}PO_4$, insulin or ACTH caused phosphorylation of the particulate phosphodiesterase (eluted from DEAE columns after solubilization as described above).

Significance to Biomedical Research:

It seems that the phosphodiesterases of many tissues are under complex control by several factors, including hormones and their importance in the regulation of cyclic nucleotide metabolism is becoming increasingly apparent.

Proposed Course:

To further characterize the high affinity particulate phosphodiesterase of rat adipocytes, to clarify the mechanisms whereby this enzyme is subject to control by insulin, cAMP, glucocorticoids and thyroid hormones.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00609-08 CM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Hormone Sensitive Lipase Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.-C. Tsai	Research Chemist	CM NHLI
OTHER:	M. Vaughan	Chief, Laboratory of Cellular Metabolism	CM NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

SUMMARY OF WORK (200 words or less - underline keywords)

Work on purification of the hormone-sensitive lipase and characterization of the lipase inactivation system has been largely suspended during the past year but will be resumed in the future.

PHS-6040
(12-75)

Project Description:

Objectives. (1) To purify the fat cell hormone-sensitive lipase and the enzymes that regulate its activity, and (2) to elucidate the mechanisms of lipase activation and inactivation.

Methods. Assay of lipase activity using ^{3}H-glyceryl trioleate as substrate, precipitation of the unhydrolyzed substrate with 5% trichloroacetic acid and radioassay of ^{3}H-glycerol in the supernatant fluid. Fractionation and purification of hormone-sensitive lipase using ammonium sulfate precipitation, gel chromatography and disc gel electrophoresis.

Major Findings. As we found several years ago, most of the hormone-sensitive lipase in soluble fractions from rat adipose tissue is precipitated with ammonium sulfate at 45% saturation. We have now compared the characteristics of the lipase activity in this fraction with those of the lipase that is precipitated only at higher concentrations of ammonium sulfate and have found that they differ in several respects. The effects of assay conditions (e.g., buffer, cations) have been defined. The results of these and earlier studies are being prepared for publication.

Significance to Heart and Lung Research:

Availability of plasma FFA, an important energy substrate for heart, is regulated via the hormone sensitive lipase activity of adipose tissue. Purification of the lipase and the inactivating enzyme are essential before the mechanisms through which its activity is regulated can be elucidated.

Proposed Course:

Work on this project has been suspended for most of the past year in order to devote more effort to other projects. It will be resumed in the future as permitted by consideration of the overall program and resources of the laboratory.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00610-01 CM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Phosphodiesterase Activity by Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter S. Ross	Staff Associate	CM NHLI
OTHER:	Martha Vaughan	Chief, Lab. Cellular Metabolism	CM NHLI
	V. C. Manganiello	Medical Officer (Research)	CM NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

The subject of this project is the hormonal regulation of cyclic nucleotide levels and cyclic nucleotide phosphodiesterase activity, with special emphasis on cyclic guanosine 3',5'-monophosphate (cGMP). The effect of the steroid dexamethasone on cyclic guanosine monophosphate levels and cyclic guanosine monophosphate phosphodiesterase activity in HTC tissue culture cells and rat liver and other organs is under investigation.

PHS-6040
(12-75)

Project Description:

Objectives. To define the hormonal regulation of cyclic guanosine 3',5'-monophosphate (cGMP) phosphodiesterase and to establish the role of cGMP in mammalian liver.

Methods. Phosphodiesterase is measured by methods previously developed in this laboratory. cGMP is measured by modification of the commercially available radioimmunoassay. Phosphodiesterase is purified chromatographically. Hepatoma cells are grown using routine tissue culture methods.

Major Findings. Incubation of hepatoma (HTC) cells with 1 μM dexamethasone for 48-72 hr resulted in a 50% decline in cGMP phosphodiesterase activity. In the continued presence of dexamethasone the depression of phosphodiesterase activity persisted for several days. Incubation with dexamethasone did not alter the cGMP content of HTC cells.

The cGMP phosphodiesterase, measured at a substrate concentration of 0.5 μM, is confined to the 100,000 g supernatant fraction of HTC cells. Chromatography on DEAE-Biogel resolves this activity into two peaks, one with high affinity for cGMP (K_m ∿1 μM) and the other with a lower affinity for cGMP (K_m >50 μM). Activity in both peaks was decreased after incubation of HTC cells with dexamethasone. A similar effect of dexamethasone was demonstrated *in vivo*. Rats treated for 72 hr with pharmacologic doses of dexamethasone had diminished cGMP phosphodiesterase activity in liver. In lung, cGMP phosphodiesterase activity was also significantly decreased by treatment of rats with dexamethasone.

Significance to Heart and Lung Research:

cGMP may play an important role in contraction of smooth and cardiac muscle. Cyclic nucleotide degradation appears to be regulated in a complex fashion in many tissues.

Proposed Course:

The phosphodiesterases will be further purified. The mechanism of the dexamethasone-induced decline in phosphodiesterase activity will be further investigated. The role of cGMP in liver will be explored.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00611-01 CM

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Membrane Microviscosities of Human Skin Fibroblasts.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Avigan	Research Chemist	CM NHLI
	C. Chignell	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

The microviscosities of cell membranes of normal and type II homozygous hyperlipoproteinemia skin fibroblasts, as well as the effect of incubation with serum lipids thereon, have been studied employing electron spin resonance and fluorescent polarization techniques.

PHS-6040
(12-75)

Background and Objectives:

The structure and composition of cellular membranes affect the physical rigidity of their microenvironment. In particular, increasing concentration of sterol in the lipid phase of biological membranes is known to reduce membrane fluidity. There are numerous indications in literature that transformed and tumor-like cells have different plasma membrane viscosities than their diploid counterparts. The reduced feedback response of cholesterol synthesis to exogenous lipoproteins in the transformed cells may parallel certain differences in their membrane structure and composition. The most striking difference between similar cells with regard to their biochemical responses to low density lipoproteins is that between normal human fibroblasts and those derived from type II hyperlipemic individuals. In the latter there is little or no feedback control of cholesterol synthesis, a phenemonon correlated with deficient binding to, and/or transport of the lipoproteins through the plasma membrane. These processes could be influenced by genetically controlled differences in membrane composition. Furthermore, the deficiencies in metabolic interactions with the cholesterol-carrying molecules could conceivably produce plasma membranes of different physical properties. The present study was designed to determine whether such differences were demonstrable.

Methods:

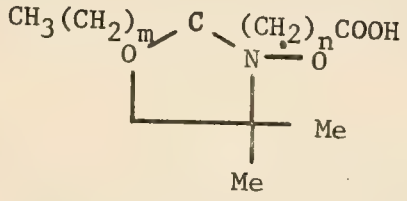
Cells were grown to confluency. In some experiments cultures were incubated in a lipid deficient medium before harvesting. Labeling with electron spin resonance (ESR) or fluorescent polarization probes was carried out before washing and harvesting cells by scraping or trypsinization.

Electron spin resonance (ESR) spectra were recorded with a Varian E-4 spectrometer operating at 9.5 GHz. Samples were introduced into the microwave cavity in a flat quartz microcell. The samples were maintained at constant temperature. Polarization measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with Glan-Thompson prisms. The temperature of the fluorescence cell was maintained with a constant temperature bath.

Findings:

The fluorescence polarization of 1,6-diphenylhexatriene bound to the membranes of the two cell lines is shown in Table 1. Some difficulty was experienced in preparing a cell suspension free of large aggregates which would remain suspended during the polarization measurements. Cells which had been mechanically removed from the culture flasks then gently homogenized showed almost the same polarization value irrespective of their source. By contrast, when trypsin was employed the normal cells had slightly greater cell membrane viscosity. The relatively small differences between the cells do not appear to be significant.

The cell membranes of the two cell lines were also examined with the aid of three stearic acid probes bearing the stable nitroxide radical (I-III).



I	m = 12	n = 3
II	m = 5	n = 10
III	m = 1	n = 14

Each of the above probes could be expected to yield a measure of microviscosity at different membrane depth.

No differences could be detected with any of these probes between normal and homozygous type II cell lines whether exposed to medium containing whole serum or to lipid-free medium.

The ESR spectrum of label I bound to the cells was recorded over a temperature range from 5° to 40°. When the maximal hyperfine splitting ($2T_{11}$) of label I was plotted as a function of temperature a shallow break was observed at about 24-25° for both types of cells. If such a discontinuity is indicative of a phase change in the membranes, then it would appear that both cell lines exhibit the same transition temperature.

Table 1

Fluorescence polarization of 1,6-diphenylhexatriene bound to cell membranes of normal and homozygous type II lines.

Exp.	Cells	Method of cell suspension	Temp. of meas.	Polarization (+ SEM)
1	Control	trypsinization	37°	0.170 ± 0.005
	Type II	"	"	0.157 ± 0.003
2	Control	homogenization	"	0.148 ± 0.005
	Type II	"	"	0.154 ± 0.003
3	Control	"	25°	0.175 ± 0.004
	Type II	"	"	0.165 ± 0.004
	"	sonification	"	0.158 ± 0.008

Significance to Heart and Lung Research:

Plasma membrane has an important role in the transport of lipids between the cell and its environment. The present study clearly demonstrates that the physiological abnormality in type II hyperlipemia is not associated with any abberation in the fluidity of the plasma membrane demonstrable by the physical methods applied in our experiments.

Proposed Course:

It is planned to carry out some additional measurements of membrane fluidity in cells that underwent treatments affecting their lipogenic activity.

Annual Report of the
LABORATORY OF CHEMICAL PHARMACOLOGY
National Heart and Lung Institute
July 1, 1975 through June 30, 1976

DRUG METABOLISM AS A CAUSE OF DRUG TOXICITY

During the past few years this laboratory has developed an integrated approach for determining 1) whether various toxicities caused by different organic compounds are mediated through the formation of chemically reactive metabolites that combine covalently with various tissue components, 2) whether there is a dose threshold for the toxicity and the covalent binding of the chemically reactive metabolites, 3) the mechanisms by which the reactive metabolites are formed and inactivated, 4) whether enzymes present in a given tissue accounts for all of the metabolites that are covalently bound to tissue macromolecules or whether they are also formed in other tissues, 5) the mechanisms by which various treatments alter the formation and elimination of the reactive metabolites and thereby change the incidence and severity of the toxicity and 6) the conditions under which the extrapolation of data obtained in vitro to living animals will be reasonably valid and when it won't. With our approach, the laboratory has discovered that the liver necrosis caused by chemically reactive metabolites of halogenated benzenes, acetaminophen and furosemide in animals occurs only after threshold doses are exceeded and that these dose thresholds are due to dose dependent changes in the metabolism of the toxicants. It has also been discovered that the incidence of liver necrosis caused by other drugs and foreign compounds, including carbon tetrachloride, isoniazid and iproniazid and the covalent binding of their reactive metabolites do not depend on a threshold dose but are approximately proportional to the dose of the toxicant. In addition, the laboratory has discovered why a given treatment may increase the toxicity of a drug in one animal species and decrease it in another by causing different effects on the relative activities of drug metabolizing enzymes. We are thus gaining a better understanding of the mechanisms by which organic compounds cause tissue lesions and of the effects of various treatments and different dosage schedules on drug-induced toxicities.

Acetarylamides

Acetaminophen toxicity - In previous studies, it was found that the centrilobular liver necrosis caused by high doses of acetaminophen was mediated by a chemically reactive metabolite formed by cytochrome P-450 enzymes in liver endoplasmic reticulum. At therapeutic doses of the drug, the reactive metabolite combines with glutathione both enzymatically and nonenzymatically and thus little covalent binding and no liver necrosis occurs. At high doses, however, virtually all of the glutathione in the liver is consumed after which the reactive metabolite then combines with tissue macromolecules and liver necrosis ensues. Because cysteamine combines nonenzymatically with the reactive metabolite and may partially prevent its formation, cysteamine has been used as an antidote in patients poisoned with acetaminophen. But cysteamine causes serious side effects, including hypotension, nausea and general lethargy. It may be important, therefore, that glutathione administered intravenously also decreases the depletion of glutathione in liver and thereby prevents

liver necrosis.

In some patients, large doses of acetaminophen also causes necrosis in the proximal tubules of the kidney. However, the appearance of the kidney lesion in animals depends on the relative activities of the cytochrome P-450 in liver and kidney and on the mode of administration of the drug. In mice and hamsters the activity of the enzyme is much higher in liver than in kidney and thus only liver necrosis occurs after lethal doses of the drug. In rats, however, the activity of the enzyme in liver is low and thus large doses of acetaminophen (> 1000 mg/kg) administered subcutaneously cause kidney damage as well as liver necrosis.

Phenacetin toxicity - Large doses of phenacetin cause liver necrosis in man. In animals, however, there is a marked species difference in the hepatotoxicity of phenacetin. Large doses (900-1200 mg/kg) of the drug causes no liver necrosis in rats and only occasionally cause liver necrosis in mice. In hamsters, however, phenacetin causes extensive liver necrosis especially in animals pretreated with 3-methylcholanthrene, which induces the enzyme that converts phenacetin and acetaminophen to chemically reactive metabolites.

The mechanisms that convert phenacetin to chemically reactive metabolites differ markedly from those that activate acetaminophen. The pretreatment of hamsters with 3-methylcholanthrene increases the covalent binding of acetaminophen *in vitro* but decreases that of phenacetin. On the other hand, the pretreatment of hamsters with phenobarbital increases the covalent binding of phenacetin *in vitro* but has little effect on that of acetaminophen. Moreover, the glutathione conjugate formed from phenacetin in the presence of liver microsomes, NADPH and $^{18}O_2$ no longer contains an ethoxy group but does contain ^{18}O in the phenolic group. But the glutathione conjugate formed from acetaminophen does not contain ^{18}O. Thus phenacetin is apparently converted to a 3,4-epoxide either before or after O-dealkylation whereas acetaminophen is apparently activated by another mechanism, presumably by an N-hydroxylation reaction followed by rapid conversion to a quinimide. Phenacetin is also converted to N-hydroxyphenacetin which is relatively stable until it is converted to either its N-O-sulfate or its N-O-glucuronide metabolites by liver enzymes. Again, however, the glutathione conjugates formed from these metabolites no longer possess the ethoxy group indicating that the metabolites undergo a deethylation as they decompose to their reactive metabolites.

N-Hydroxy-2-acetylaminofluorene (NOH-AAF) - This carcinogen is thought to evoke its carcinogenic activity through the formation of its NO-acetyl, NO-sulfate or N-O-glucuronide. In accord with this view, the conversion of NOH-AAF to its sulfate derivative greatly increases its covalent binding to tissue macromolecules *in vitro*. However, the chemical reactivity of the reactive metabolite derived from the sulfate conjugate of NOH-AAF differs from the chemical reactivity of sulfate conjugate of N-hydroxyphenacetin. The sulfate conjugate of NOH-AAF apparently reacts with glutathione, methionine, adenosine and guanosine because these substances block the covalent binding of the conjugate to tissue proteins, By contrast, the covalent binding of the sulfate conjugate of N-hydroxyphenacetin to liver protein is blocked by glutathione but not by the other three substances. The inability of the sulfate conjugate of N-hydroxyphenacetin to react with guanosine and adenine may account for its

lack of carcinogenic potential.

Although the sulfate conjugate of NOH-AAF is highly chemically reactive and can react with purine bases, it is not the most mutagenic metabolite of NOH-AAF as measured by the TA-1538 tester strain of S. typhimurium constructed by Ames. An enzyme in the soluble fraction of rat liver markedly increases the mutagenicity of the NOH-AAF even after all cofactors have been removed from the preparation. Addition of the cofactors required for sulfation of NOH-AAF, however, markedly decreases the mutagenic index. Thus we are on the horns of a dilemma; either the sulfate conjugate of NOH-AAF is not as carcinogenic as it is thought to be, or there is no relationship between the mutagenicity index of a substance as measured by the Ames test and its potency as a carcinogen.

Hydrazines

Isoniazid - Previous studies indicate that this drug causes hepatitis in humans through the following sequence of events: Isoniazid is first acetylated to form acetylisoniazid which then is hydrolyzed to form acetylhydrazine and isonicotinic acid. Acetylhydrazine is then N-hydroxylated by a cytochrome P-450 enzyme in liver microsomes to form a highly chemically reactive metabolite. Although the identity of the reactive metabolite remains unknown, it cannot be ketene. In addition to being converted to the reactive metabolite, acetylhydrazine is also acetylated in the body to form diacetylhydrazine. This metabolite, however, is nontoxic and is not converted to a chemically reactive metabolite either in vivo or in vitro.

Nitro compounds

Nitrofurantoin - This drug used in the treatment of urinary tract infections, has caused serious and potentially fatal lung damage in patients. In pathogen free rats large doses (100-400 mg/kg) of nitrofurantoin cause lung damage manifested by hemorrhages, edema and necrosis. Covalent binding of the drug also occurs in kidney liver and lung. In vitro studies indicate that under anaerobic conditions if nitrofurantoin as well as many other nitrofuran drugs is converted to reactive metabolites by several enzymes including aldehyde oxidase, xanthine oxidase and liver microsomal NADPH cytochrome c reductase. But whether the lung damage is caused by a reactive metabolite remains to be determined.

Last year we reported that the conversion of the nitrobenzene to metabolites that cause methemoglobinemia in vivo is catalyzed almost entirely by gut flora rather than by mammalian nitroreductases. However, the treatment of rats with antibiotics does not decrease the toxicity of nitrofurantoin or 1-nitronaphthalene. Moreover, the covalent binding of nitrofurazone, another nitrofuran drug, occurred as rapidly in germ-free mice as it does in mice acclimatized to laboratory conditions. Since the reduction of nitrobenzene by liver homogenates under anaerobic conditions is catalyzed mainly by cytochrome P-450 it is evident that the relative roles of bacterial flora and the mammalian tissues in activating nitro compounds depends on the substrate specificities of the xanthine oxidase and aldehyde oxidase.

5-Membered aromatic heterocyclic compounds

In previous years we have discovered that high doses of several furans caused tissue necrosis in liver, lung and kidney of mice and rats. It is now apparent that several thiophenes may also cause tissue necrosis. For example, thiophene causes primarily hepatic necrosis whereas 2-thenamide and 2-ethylthenoate cause both hepatic and renal damage.

Cephaloridine - Large doses of this thiophene derivative used as an antibiotic causes renal necrosis in mice, rats and rabbits. The findings that piperonyl butoxide and $CoCl_2$ prevent the necrosis diethyl maleate potentiates the toxicity and that cysteine decreases it suggest that the metabolite may be chemically reactive. In mice diethyl maleate shifts the zone of necrosis in the proximal tubules from the outer cortex of the mid-inner cortex. In rats, however, cysteine shifts the zone of necrosis from the outer to the inner cortex.

Ipomeanol - This furan compound, present in moldy sweet potatoes, causes pulmonary edema in cattle. In rats, it initially causes a thickening of the alveolar walls and swelling of type II cells. As the damage progresses, the lungs become edematous and eventually the normal integrity of the lung is lost and widespread multicellular necrosis, hemorrhage and consolidation occurs. These toxic effects are probably mediated by a chemically reactive metabolite of 4-ipomeanol. After the injection of radiolabeled 4-ipomeanol to rats, the concentration of glutathione decreases and a covalently bound metabolite appears in lung within about 2 hr; the binding is greatest in the bronchiolar lining cells and alveolar type II cells. Moreover, pretreatments of rats that alter the metabolism of ipomeanol or affect the concentration of glutathione in lung cause parallel effects on the severity of the toxicity and the magnitude of the covalent binding. Studies in vitro indicate that the reactive metabolites may be formed not only in lung but also in liver and may combine with glutathione, cysteine and a number of mercaptans to varying degrees as well as to microsomal protein.

There are marked species and strain differences in the toxicity of ipomeanol. For example, the LD_{50} values in various species were 15 mg/kg in guinea pigs, 24 mg/kg in HLA-SD rats, 30 mg/kg in rabbits, 20-60 mg/kg in various strains of mice and 140 mg/kg in hamsters. Moreover, tissue necrosis occurs only in the lung of rats, guinea pigs and rabbits, in both the lung and the kidney of mice and both the liver and the lung of hamsters. The covalent binding of ipomeanol to tissue proteins is greatest in the lung of rats, in the kidney of mice and in the liver of hamsters. Moreover, the pretreatment of C57BL/64 mice with 3-methylcholanthrene, which increases cytochrome P-450 enzymes in liver, increased the covalent binding of ipomeanol to tissue protein in liver but decreased it in lungs and kidney. As a result, the pretreatment with 3-methylcholanthrene shifted the toxicity from lung and kidney to liver and increased the LD_{50} from 25 mg/kg to 125 mg/kg. These findings suggest that the reactive metabolite of ipomeanol is so highly reactive that it does not escape the organ in which it is formed.

Repetitive administration of low doses (5-8 mg/kg) of ipomeanol for 7 days

increases the LD_{50} of ipomeanol in rats. The development of this tolerance may be due not only to a decrease in the formation of the chemically reactive metabolite, but it also may be due to other mechanisms. The repetitive administration of low doses (5 mg/kg) of ipomeanol, which produces tolerance but no apparent alteration in cytochrome P-450, causes a marked proliferation of type II pneumocytes and an increase in the number of their lamellar bodies, which contain surfactant, and squamous metaplasia of the bronchiolar lining cells.

Halogenated compounds

Trichloroethylene and dichlorovinylcysteine. Trichloroethylene-treated soybean meal contains dichlorovinylcysteine that causes aplastic anemia when fed to calves. We have found that trichloroethylene administered orally to calves causes areas of partial aplasia and hypoplasia, which may result eventually in aplastic anemia; thus it seems possible that dichlorovinylcysteine may be formed *in vivo*.

Chloramphenicol - Last year we reported that the covalent binding of chloramphenicol in rats probably occurs through the activation of the dichloroacetyl-moiety of the drug rather than through the chloramphenicol base. In support of this view, we have found that the oxalic acid analogue is formed by rat liver microsomes *in vitro* and that acid hydrolysis of covalently bound chloramphenicol results in the chloramphenicol base and oxalic acid. Furthermore, when the hydrogen of the dichloroacetyl moiety is labeled with tritium, the label is not present in the covalently bound chloramphenicol; thus we believe that chloramphenicol is first hydroxylated to form a highly unstable dichlorohydroxyacetyl derivative that rearranges to form an oxalyl chloride which in turn acylates tissue proteins. In accord with this view, the trifluoroacetyl analogue of chloramphenicol does not covalently bind to tissue proteins either *in vivo* or *in vitro*.

In calves, chloramphenicol causes highly localized areas of aplasia which may be characteristic of aplastic anemia. It seems possible, therefore, that calves might serve as a model for chloramphenicol-induced aplastic anemia. Further studies are underway to evaluate this possibility.

Other toxic compounds

α-Methyldopa - Last year we reported that the covalent binding of α-methyldopa in the presence of rat liver microsomes and NADPH was due largely to the oxidation of the catechol by superoxide and that the superoxide was formed mainly by cytochrome P-450. In addition, however, α-methyldopa is covalently bound to intact and lyzed red blood cells by a mechanism that is not entirely clear, because the covalent binding is inhibited by CO and N_2 but not by superoxide dismutase. Whether the covalent binding of α-methyldopa mediates the toxic effects of the drug in patients remains to be investigated.

Paraquat - This herbicide causes edema and necrosis in pulmonary alveoli followed by interstitial fibrosis and death. In previous years, we have reported that the lethal effects of paraquat may be partially prevented by the β-adrenergic blocking agent, propranolol, and potentiated by the β-adrenergic

agonist, isoproterenol. During the past year, we found that paraquat toxicity was potentiated by several different β-2 adrenergic agonists (salbutamol, carbuterol and fenoterol) as well as l-isoproterenol. Pretreatment of rats with the propranolol completely prevented the potentiating effects of the agonists. But the potentiation of the toxicity by l-isoproterenol was not blocked by β-l adrenergic blocking agents. Thus it appears that the potentiating effect of the adrenergic drugs involves β-2 adrenergic receptors.

Studies on the mechanism by which isoproterenol potentiates paraquat toxicity suggest that isoproterenol decreases the clearance of paraquat presumably by slowing the renal blood flow. In accord with this view, isoproterenol decreases the clearance of both N-methyl nicotinamide and p-aminohippurate. However, a part of the effect of isoproterenol may occur through another mechanism, because dopamine, which increases renal blood flow, failed to prevent the potentiation of paraquat toxicity by l-isoproterenol.

Since paraquat is thought to exert its toxicity by producing superoxide, we have studied the effects of paraquat on the covalent binding of radiolabeled norepinephrine. Although the covalent binding of norepinephrine by microsomes of liver and lung is mediated by superoxide, paraquat did not increase the covalent binding of norepinephrine by lung slices. Moreover, sodium cyanide, which inhibits superoxide dismutase, and paraquat did not produce malondialdehyde in lung slices. The mechanism by which paraquat causes lung toxicity thus remains obscure.

PHYSIOLOGICAL CONTROL MECHANISMS

Cyclic nucleotides and amylase secretion by pancreas - In pancreatic slices from guinea pigs, amylase secretagogues, carbamylcholine and cholocytokinin octapeptide increase both the concentration of cyclic GMP and the secretion of amylase but do not change the concentration of cyclic AMP. Thus, cyclic GMP apparently mediates the secretion of amylase.

The secretagogues evoke their effects by different mechanisms. Although atropine blocks the action of carbamyl choline, it did not inhibit the effects of either caerulin or cholocytokinin octapeptide.

Cyclic nucleotides and lipolysis - Although epinephrine increases both cyclic AMP and cyclic GMP in fat cells, epinephrine-induced lipolysis is apparently mediated only by cyclic AMP. The role of cyclic GMP in adipocytes thus remains obscure.

Cyclic GMP and platelet aggregation - Guanylate cyclase activity in blood platelets can be increased by arachidonic acid (5-10 μM) and the increase can be blocked by anerobic conditions or by indomethacin, an inhibitor of cyclooxygenase. Moreover, phospholipase A_2, which releases arachidonate from phospholipids also increases the guanylate cyclase activity. Thus, the guanylate cyclase is apparently activated by a metabolite of arachidonate, but whether the metabolite is thromboxane, one of the prostaglandins, or another intermediate remains to be determined.

Treatment of essential hypertension with propranolol - Because propranolol lowers plasma renin activity in patients, it has been suggested that the decrease in renin accounts for the antihypertensive effects of the drug. A study of 25 patients with essential hypertension revealed, however, that plasma renin activity was maximally lowered at doses of propranolol (80 mg) that do not lower blood pressure in most patients. Most of the patients required 160-320 mg of propranolol to control their blood pressure. After several weeks at these doses, the blood pressure began to increase, but the increase could be prevented by a diuretic combined with propranolol.

Current analytical methods for assaying propranolol and its active metabolites in plasma are nonspecific and insensitive. We are assessing several different methods developed in the laboratory based on 1) gas chromatography, 2) high pressure liquid chromatography or 3) combined gas chromatography-mass spectrometry and selective ion monitoring.

Search for unidentified mineralocorticoids - Because both aminoglutethimide, an inhibitor of steroid biosynthesis, and spironolactone, an antagonist of mineralocorticoid action, decrease the blood pressure in low renin essential hypertensive patients, it has been suggested that the disease is caused by an excess of an unknown mineralocorticoid, possibly 16β-hydroxy-dehydroepiandrosterone (16β-OH-DHEA) or 18-hydroxy-DDC. However, studies utilizing an assay method for 16β-OH-DHEA developed in our laboratory, indicated that aminoglutethimide lowered the blood pressure without having a consistent effect on the excretion of 16β-OH-DHEA. Thus, the higher plasma concentration of the steroid probably does not account for the hypertensive state of the patient.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00803-07 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Role of Cytochrome b_5 in Cytochrome P-450 Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	H.A. Sasame	Chemist	LCP NHLI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	0.4

SUMMARY OF WORK (200 words or less - underline keywords)

Simultaneous determinations of oxygen uptake, the oxidation of NADPH and NADH, and ethylmorphine by liver microsomes isolated from normal, phenobarbital and pregnenolone 16α-carbonitrile pretreated rats revealed that a part of oxidation of NADH results in the formation of an unstable active oxygen species, but does not result in the reduction of this species. These findings indicate that the role of cytochrome b_5 is closely intertwined with the functional state of NADPH-mediated P-450 system as a source of the second electron in drug metabolism and the formation of the active oxygen species.

PHS-6040
(12-75)

Project Description:

Objectives: Last year we reported the results of immunochemical studies which demonstrated that cytochrome b_5 participates not only in the synergistic effect of NADH on NADPH-mediated P-450 reactions but also in NADPH-mediated reactions. It was particularly noteworthy that anticytochrome b_5 raised the rate of superoxide anion in mixtures containing liver microsomes isolated from phenobarbital pretreated rats, ethylmorphine and NADPH, while partially inhibiting ethylmorphine metabolism. The object of this project was to delineate further the interrelationship between the formation of superoxide anion and function of P-450 in liver microsomes.

Methods Employed: An anticytochrome b_5 antibody fraction was prepared from antiserum of sheep immunized against cytochrome b_5 purified from rat liver microsomes as reported in last year's report. The rate of superoxide anion formed by cytochrome P-450 enzymes in rat liver microsomes was assayed spectrophotometrically by following the rate of adrenochrome from epinephrine. Standard biochemical procedures were used to measure other functional components of the microsomal cytochrome P-450 system.

Major Findings: We have estimated the minimal fraction of the oxygenated cytochrome P-450 that is reduced by cytochrome b_5, in microsomal systems isolated from normal, phenobarbital (PB) and pregnenolone-16α-carbonitrile (PCN) pretreated male rats. The fraction was significantly lower in PCN microsomes than in normal and PB microsomes. This finding agrees with our previous report that PCN markedly retards the reduction of cytochrome b_5 by both NADPH and NADH.

Simultaneous determinations of oxygen uptake, the oxidation of NADPH and NADH, and ethylmorphine metabolism in PCN microsomes revealed that there is a 1:1 stoichiometric relationship between the pyridine nucleotide oxidation and oxygen uptake but not between these values and the metabolism of ethylmorphine. These observations suggest that a part of the oxidation of NADH results in the formation of an "unstable active oxygen species" which could be either superoxide or hydrogen peroxide. However, the hydrogen peroxide must oxidize another component in liver microsomes. The irreversible nature of "active oxygen species" was also substantiated by the fact that the addition of methanol and catalase, which leads to the formation of formaldehyde, failed to alter the rate of either oxygen uptake or the oxidation of NADPH and NADH.

We also measured the amount of acetaldehyde formed by the N-deethylation of lidocaine, and the amount of formaldehyde formed by the catalase and methanol system with PB and PCN microsomes and NADPH. Although the anticytochrome b_5 antibody did not affect the rate of NADPH, it decreased the rate of N-deethylation of lidocaine and increased the rate of formaldehyde formation. However, the addition of NADH to a system consisting of NADPH, liver microsomes, lidocaine, catalase and methanol causes an increase in the

N-deethylation of lidocaine but does not increase the formation of formaldehyde.

Significance to Biomedical REsearch and the Program of the Institute: Since it has been suggested that superoxide may cause cytotoxic effects, it is important to delineate the role of cytochrome b_5 in monitoring the level of superoxide anion.

Proposed Course of Project: We shall continue to study the effect of the anticytochrome b_5 antibody on the *in vitro* metabolism of various drugs and steroids that cause toxicity in animal models.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00804-07 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the Detoxification of Reactive Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Borner	Visiting Fellow	LCP NHLI
	J.A. Hinson	Staff Fellow	LCP NHLI
	G.J. Mulder	Guest Worker	LCP NHLI
	J.R. Gillette	Chief, Laboratory of Chemical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Mulder is a Guest Worker from the Department of Pharmacology, State University of Groningen, The Netherlands.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug-Interaction

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

SUMMARY OF WORK (200 words or less - underline keywords) Many drug- or chemical-induced toxicities to various organs as lung, kidney, and liver occur by metabolism of the parent compound to reactive electrophilic metabolites. Electrophilic metabolites have also been implicated in chemical carcinogenesis. A role for the nucleophile glutathione (GSH) in detoxification has been established with the reactive metabolites of bromobenzene and acetaminophen, but not with other toxicities known to be mediated through electrophilic metabolites. The role of GSH transferase in detoxification of reactive metabolites is uncertain. A simple in vitro assay has been designed to study the possible role of GSH and GSH transferase in the detoxification of reactive metabolites. In this assay the reactive metabolites are generated enzymatically and the detoxification with nucleophiles such as glutathione, cysteine and methionine studied by direct addition to the incubation mixture. The effect of glutathione transferases was determined by addition of these enzymes to the incubation mixtures plus GSH. It has been determined that at 500 μM GSH the reactive metabolite of acetaminophen is very effectively detoxified nonenzymatically. At 50 μM GSH, however, the reactive metabolite is not effectively detoxified, but after addition of GSH transferase the reactive metabolite is completely detoxified. A similar role for GSH transferase in the detoxification of the reactive metabolites of furosemide, bromobenzene and naphthalene has been established.

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Project Description:

Objectives: Previous studies in this laboratory have shown that the hepatic necrosis observed with acetaminophen, furosemide, bromobenzene and isoniazid have resulted from the formation of electrophilic reactive metabolites. Reactive metabolites have also been implicated in other toxicities such as lung edma, nephrotoxicity and carcinogenesis. A role for the nucleophile glutathione in detoxification has been established with the reactive metabolites of bromobenzene and acetaminophen, but not with other toxicities known to be mediated through reactive electrophilic metabolites. The role of glutathione transferases is unknown. We thus have examined a role for glutathione and the enzyme glutathione transferase in the detoxification of certain selected reactive metabolites. Also we have examined the potential of these reactive metabolites to bind to other tissue nucleophiles as cysteine, methionine, guanine and adenine.

Methods Employed: Reactive metabolites were enzymatically generated. Both microsomal reactive metabolites and reactive metabolites formed following sulfation or glucuronidation were studied. The nonenzymatic detoxification by potential tissue nucleophiles such as glutathione and cysteine was determined by direct addition of these nucleophiles to the incubation. The capacity for enzymatic detoxification was studied by addition of appropriate enzyme fractions which had been chromatographed on Sephadex G-25. All other methods were standard procedures.

Major Findings: Addition of varying concentration of glutathione to microsomal incubations containing acetaminophen and cofactors, for generation of the reactive metabolite, showed covalent binding to macromolecules decreased to almost zero at 0.5 mM glutahione. When 100,000 x g supernatant which had been chromatographed on Sephadex G-25 was added, the same decrease in covalent binding occurred at a concentration of 0.05 mM glutathione. There was corresponding increase in the formation of an acetaminophen glutathione conjugate. A similar role for glutathione transferase has been established in the detoxification ofthe reactive metabolites of furosemide, bromobenzene, and naphthalene. The nonenzymatic detoxification reaction proceeded equally well with glutathione, cysteine or N-acetylcysteine but not at all with methionine or s-methylglutathione. The enzymatic detoxification of the reactive metabolite of acetaminophen was ineffective with other nucleophiles as cysteine, N-acetylcysteine, methionine and S-methylglutathione. Glutathione was only partially effective, however, in blocking the covalent binding of the sulfate of N-hydroxy-2-acetylaminofluorene, the presumed proximate carcinogenic metabolite of 2-acetylaminofluorene. Methionine, however, blocked the covalent binding of this reactive metabolite quite effectively as did guanine and adenine in contrast to the reactive metabolites of the monocyclic N-arylacetamide.

Significance to Biomedical Research and to the Program of the Institute: The present studies demonstrate a role for glutathione transferase in detoxification of some reactive metabolites.

Proposed Course of Project: Since studies on the enzymatic and non-enzymatic detoxification of reactive metabolites are important in studies on the toxicity of drugs and other chemicals. These studies will be continued with other reactive metabolites.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00806-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Activation of N-Hydroxy-N-Arylacetamides by Sulfation and Glucuronidation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gerard J. Mulder	Guest Worker	LCP NHLI
OTHER:	Jack A. Hinson	Staff Fellow	LCP NHLI
	James R Gillette	Chief, Laboratory of Chemical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Mulder is a Guest Worker from the Department of Pharmacology, State University of Groningen, The Netherlands.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms of nephrotoxicity, hepatotoxicity, hemolysis, methemoglobinemia, and carcinogenesis of N-arylacetamide are believed to occur through metabolism. The key metabolite in each of these toxicities has been postulated to be the N-hydroxy derivative. N-hydroxylation of the analgesic acetaminophen is believed to produce hepatotoxic metabolites. N-hydroxylation of polycyclic N-arylacetamides followed by N-O-sulfation is believed to produce the ultimate carcinogenic metabolite of these compounds. Since we have previously shown that the analgesic phenacetin and related compounds are readily N-hydroxylated, we have examined the capacity of these compounds to be sulfated and glucuronidated. Thus N-hydroxyphenacetin is readily sulfated to form an electrophilic metabolite which covalently binds to macromolecules. This compound is also glucuronidated to form an electrophlic metabolite. Other phenacetin analogs were also sulfated and glucuronidated but the metabolites were not electrophilic. The conditions for the activation and the detoxification of these reactive metabolites have been studied.

Project Description:

Objectives: The mechanisms of nephrotoxicity, hepatotoxicity, hemolysis and methemoglobinemia of the monocyclic N-arylacetamides are unknown. In previous studies we have shown that phenacetin and p-chloroacetanilide are N-hydroxylated by a microsomal cytochrome P-450 mixed functions oxidase. Since N-O-sulfation and N-O-glucuronidation have been implicated in the carcinogenicity and hepatotoxicity of certain polycyclic N-arylacetamides such as 2-acetylaminofluorene (through the formation of electrophilic metabolite), we examined the ability of monocyclic N-aryacetamides to be sulfated and glucuroniated.

Methods Employed: ^{14}C-N-hydroxy-N-arylacetamides were synthesized following previously described procedures. The sulfotransferase enzyme necessary for sulfation was prepared by chromatography 100,000 x g supernatant on a Sephadex-G-25 column. The glucuronyl transferase enzyme was prepared by activating microsomes with triton X-100. All other methods were standard procedures.

Major Findings: An assay has been developed for quantitating the enzymatic sulfation of these N-hydroxy derivatives. In the assay the sulfate of p-nitrophenylsulfate is transferred enzymatically to phosphoadenosyl phosphate to form the phosphoadenosyl phosphosulfate. This cofactor, which is necessary for sulfation, is then enzymatically transferred to the N-derivatives. The formation of p-nitrophenol, which is quantitated spectrophotometrically, corresponds to the sulfation of the N-hydroxy derivative.

Using this assay, we found that N-hydroxyphenacetin was sulfated at a significant rate (2.6 nmoles per min per mg protein) by enzymes in the soluble fraction of liver. The N-hydroxyphenacetin sulfate was not a stable metabolite and was found to readily arylate macromolecules. The stoichiometry showed that the majority of the electrophilic N-hydroxyphenacetin sulfate formed was covalently bound to protein. A study of other N-hydroxy-N-arylacetamides such as N-hydroxy-p-chloroacetanilide and N-hydroxyacetanilide showed these compounds were also readily sulfated but the sulfate conjugates were not electrophilic metabolites.

N-Hydroxyphenacetin was also found to be readily glucuronidated (12 nmoles per min per mg microsomal protein). The hydroxyphenacetin glucuronide was also an electrophlic metabolite, but the rate of arylation of protein was much less than with the corresponding sulfate. Other N-hydroxy derivatives studied were also glucuronidated forming stable metabolites.

Significance to Biomedical Research and to the Program of the Institute: The present studies show that monocyclic N-hydroxy-N-arylacetamides are readily sulfated or glucuronidated. Sulfation or glucuronidation of N-hydroxyphenacetin leads to the formation of electrophilic metabolites.

Proposed Course of Project: Since glucuronidation and sulfation of N-hydroxyphenacetin leads to arylating metabolites, the importance of these metabolites in the toxicities of this compound will be examined.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00807-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the mutagenicity of N-hydroxy-N-arylacetamides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gerard J. Mulder	Guest Worker	LCP NHLI
	Jack A. Hinson	Staff Fellow	LCP NHLI
	Wendell Nelson	Guest Worker	DPB NICHHD
	Snorri Thorgeirsson	Chief, Section on Molecular Toxicology	DPB NICHHD
OTHER:	James R. Gillette	Chief, Laboratory of Chemical Pharmacology	NHLI

COOPERATING UNITS (if any)
Drs. Nelson and Thorgeirsson are members of the Developmental Pharmacology Branch, National Institute of Child Health and Human Development.
Dr. Mulder is a Guest Worker from the State University of Groningen, The Netherlands

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The mutagenicity of chemicals in certain strains of salmonella typhimurium has been shown by B. N. Ames to correlate with the carcinogenicity of these chemica in animals: 90% of the mutagens were carcinogens and 90% of the carcinogens were mutagens. Since there has been shown to be a high incidence of renal pelvic tumors in abusers of the analgesic phenacetin, we examined the potential mutagenesis of N-hydroxyphenacetin and its sulfate ester. The sulfate ester of N-hydroxy-2-acetylaminofluorene, a polycyclic analog, has been postulated to be the ultimate carcinogen of 2-acetylaminofluorene. N-hydroxyphenacetin and its sulfate ester were not mutagenic. The sulfate ester of N-hydroxy-2-acetylaminofluorene was less mutagenic than the parent compound incubated with the soluble fraction alone. This indicates that the sulfate ester of N-hydroxy-2-acetylaminofluorene may not be as important in the carcinogenesis of this compound as previously believed or that the Ames' test is less responsive to highly chemically reactive metabolites than it is to relatively stable metabolites.

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Project Description:

Objectives: A high incidence of renal pelvic tumors has been correlated with abuse of the analgesic phenacetin. The mechanism of this toxicity is unknown. Since the sulfate ester of N-hydroxy-2-acetylaminofluorene (N-hydroxy-2-AAF) has been proposed as the proximate carcinogen of 2-AAF, we investigated the possibility that the sulfate ester of N-hydroxyphenacetin was carcinogenic. The Ames test offered a simple method to test this hypotheses. In this test there is a high correlation between the bacterial mutagenesis of the chemical and its carcinogenicity.

Methods Employed: All methods employed in this study were standard procedures.

Major Findings: N-Hydroxyphenacetin and N-hydroxyphenacetin sulfate were not mutagenic in this system.

The soluble fraction of rat liver increases the mutagenicity of N-hydroxy-2-acetylaminofluorene in the absence of cofactors.

The sulfate ester of N-hydroxy-2-acetylaminofluorene, the proposed proximate carcinogen of 2-acetylaminofluorene, was less mutagenic than N-hydroxy-2-acetylaminofluorene incubated with the soluble fraction of rat liver.

Significance to Biomedical Research and to the Program of the Institute: The present studies demonstrate that N-hydroxyphenacetin and its sulfate ester are not mutagenic. Also the sulfate ester of N-hydroxy-2-acetylaminofluorene may not be as important in the carcinogenesis of this compound as previously believed or that the Ames test is less responsive to highly chemically reactive metabolites than it is to relatively stable metabolites.

Proposed Course of Project: After publication of these data the project will be terminated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00826-03 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Study on the Biochemical Mechanism of Drug-Induced Aplastic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Krishna	Chief, Section on Drug-Tissue Interactions	LCP NHLI
	L. Pohl	Guest Worker	LCP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Section on Drug-Tissue Interactions

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1

SUMMARY OF WORK (200 words or less - underline keywords)

The biochemical mechanism by which chloramphenicol causes aplastic anemia was investigated using various in vivo and in vitro techniques. Chloramphenicol was shown to be activated by enzyme systems present in the liver microsomes and bone marrow cells to reactive intermediates that combine covalently with tissue macromolecules. The biochemical process appears to be hydroxylation of the dichloroacetamide group in chloramphenicol resulting in the formation of an alkylating agent. Replacement of the two chlorines with hydrogen or fluorine completely prevents this activation. Moreover isolation identification of a new metabolite of chloramphenicol, namely oxamic acid of chloramphenicol, indicates that the activation is at the dichloroacetamide part of the molecule.

PHS-6040
(12-75)

Project Description:

Objectives: Previous studies from this laboratory have indicated that chloramphenicol is activated in the body to reactive intermediate(s) that bind covalently to various macromolecules in tissues. It was also shown that thiamphenicol, an analog of chloramphenicol, is activated in the body to reactive intermediates to a lesser extent. Since it has been shown that chloramphenicol, but not thiamphenicol, induces aplastic anemia in certain sensitive individuals, we had postulated that the ability of chloramphenicol to be activated in the body to reactive intermediates may be responsible for the aplastic anemia. The exact mechanism by which chloramphenicol induces aplastic anemia is not clear, but it appears to be related to its ability to bind covalently to tissue macromolecules, especially to bone marrow cells. The main objective of this study has been to understand the mechanisms of activation of chloramphenicol by which it binds covalently to tissue macromolecules.

Methods Employed: Various ^{14}C and ^{3}H labeled analogs of chloramphenicol and its metabolites were synthesized according to standard procedures. *In vitro* incubations for measurement of covalent bindings consisting of rat liver microsomes or bone marrow cells were carried out as described in previous annual reports.

For *in vitro* studies, male Sprague Dawley rats weighing 150-180 g were used. Proteins containing covalently bound molecules were hydrolyzed by pronase treatment and amino acids containing covalently bound drug was isolated by using standard chromatography techniques.

Major Findings: Both ^{3}H-chloramphenicol (^{3}H in the secondary alcohol group) and ^{14}C-chloramphenicol (^{14}C label is the dichloroacetamide group) were bound covalently to rat liver microsomes to the same extent under various incubation conditions. The covalent binding was linear with time (up to 8 min) and protein concentration (up to 4 mg/ml). It was markedly increased by pretreatment of the animals with phenobarbital. It was markedly inhibited by SKF 525A and either N_2 or $CO:O_2$ (Table 1). Studies with various fractions of liver homogenates indicated the enzymes responsible for the conversion of chloramphenicol to active intermediates which bound covalently, was located in the microsomal fraction (Table 2). These findings indicated that cytochrome P-450 catalyzes the formation of the reactive metabolite of chloramphenicol. The intermediates involved in the covalent binding of chloramphenicol to liver microsomes may be electrophyllic in nature because glutathione markedly inhibited the covalent binding; cysteine was less effective (Table 1). Chloramphenicol is probably activated *in vitro* by oxidation of its dichloroacetamide group. 1) Formation of a new metabolite--oxamic acid analogue of chloramphenicol with liver microsomes from rats pretreated with phenobarbital. Structure of the new metabolite has been proved by cochromatography in various systems with the synthetic compound and by cocrystalization of radioactivity with the synthetic compound. 2) Acid hydrolysis (6N HCl) of the microsomal protein containing covalently bound chloramphenicol (both ^{14}C and ^{3}H) resulted in the formation of

^{3}H-chloramphenicol bas and ^{14}C oxalic acid. 3) ^{3}H in the dichloroacetamide group is lost during the covalent binding of ^{3}H-chloroamphenicol (RNH $COC^{3}H\ Cl_2$). 4) No ^{3}H-chloramphenicol base was bound covalently when it was incubated with rat liver microsomes. 5) When N-acetyl or N-trifluoroacetyl analogues of chloramphenicol were incubated with rat liver microsomes they were not covalently bound. 6) Neither ^{14}C-dichloroacetic or ^{14}C-dichloroacetamide (Table 3) were covalently bound to liver microsomes *in vitro*. These findings clearly indicate that the dichloracetamide group in chloramphenicol was the center for activation and covalent binding of chloramphenicol by liver microsomes and that entire molecule appears to be essential for the activation.

Studies *in vivo* with chloramphenicol labeled in various groups indicated that ^{14}C from dichloroacetamide group of chloramphenicol was covalently bound to various tissue macromolecules to a greater extent (5-10 times) than ^{3}H-chloramphenicol (^{3}H - in the secondary alcohol group in chloramphenicol).

Although previous studies had indicated that phenobarbital pretreatment of rat increased the covalent binding of ^{14}C-chloramphenicol *in vivo*, this was not confirmed by our present studies. Instead, the pretreatment of rats with phenobarbital caused a consistant decrease in the covalent binding of the drug to various tissues *in vivo*. Liver microsomes from the same rat liver *in vitro* show a marked increase in covalent binding of chloramphenicol. The reason for the discrepancy is not certain at this time but may be due to an increase in the rate of glucuronidation of chloramphenicol in these studies.

In vivo covalent binding of chloramphenicol also involved the activation of dichloroacetamide group. Replacement of the dichloroacetamide group by a trifluoroacetamide group in chloramphenicol molecule caused a complete loss in the capacity of this compound to bind covalently *in vivo* and *in vitro*. The covalent binding of chloramphenicol *in vivo* may also be attributed to the oxidation of dichloroacetamide group resulting in a alkylating intermediate (RNH CO COCl or RNH CO COOCl) which can either react with the proteins or water resulting in either covalent binding or formation of a new metabolite, oxamic acid of chloramphenicol (RNH CO COOH). The protein containing covalently bound chloramphenicol may undergo hydrolysis *in vivo* by various amidases or peptides resulting in only the ^{14}C of the dichloroacetamide group being predominantly bound to the proteins. On the other hand, the covalent binding of dichloroacetamide may occur after primary hydrolysis of chloramphenicol in cells because dichloroacetate when administered to rats becomes bound covalently to various tissue macromolecules (Table 5). The binding of dichloroacetate was even higher than that of chloramphenicol when administered in equivilent doses.

The covalent binding of ^{14}C in dichloroacetate probably is not due to its oxidation to carbon dioxide and incorporation into newly synthesized amino acids because: 1) Incorporation of radiolabeled acetate was less than 5% of that of dichloroacetamide. 2) The covalently bound protein

after pronase treatment and thin layer chromatography indicated no incorporation of radioactivity into glycine or any dicarboxylic amino acids. 3) The radioactivity was bound to a single unknown amino acid.

The mechanism by which dichloroacetate is activated in the body remains unknown. The possibile activation by hypoxanthine-xanthine oxidase is being considered. It is also possible that dichloroacetate may be converted to some other compounds before activation of liver enzyme systems since we found dichloroacetamide can bind covalently to liver microsomes *in vitro* in contrast to dichloroacetic acid.

Significance to Biomedical Research and the Program of the Institute:

The understanding of the mechanism of the binding of chloramphenicol may aid in the development of a new antibiotic that will have the beneficial aspects of the drug, namely a broad spectrum antibiotic activity, but without the potential toxic affect of the drug, namely aplastic anemia. The understanding of the mechanism by which chloramphenicol binds covalently to tissue macromolecules may also aid in the understanding of the mechanism of toxicity produced by other halogenated hydrocarbons, namely chloroform and halothane.

Proposed Course of Project: Isolation and purification of amino acids to which chloramphenicol is bound covalently to various macromolecules will be attempted in order to prove the mechanism of covalent binding of chloramphenicol and dichloroacetic acid both *in vivo* and *in vitro*. The mechanism by which bone marrow cells are able to covalently bind chloramphenicol will also be investigated in order to understand the mechanism of activation and the type of bone marrow cells involved in chloramphenicol-induced bone marrow damage.

Publications:

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Asghar, K. Reddy, R.G. and Krishna, G.: Histochemical localization of glutathione in tissues. J. Histochem. Cytochem. 23: 774-779, 1975.

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Rao, G. S., Krishna, G. and Gillette, J. R.: Enzymatic formation of chemically reactive metabolites of N-nitrosodesmethyl tripelennamine by a mechanism other than N-dealkylation. Biochem. Pharmacol. 24: 1707-1711, 1975.

Rao, G. S., Krishna, G. and Gillette, J. R.: Metabolism, tissue distribution and covalent binding of tripelennamine and its N-nitroso derivative in the rat. J. Pharmacol. Exp. Therap. 195: 433-440, 1975.

Rao, G. S., Krishna, G. and Gillette, J. R.: Drug-nitrite interactions: The lack of toxicity of a N-nitroso derivative of tripelennamine formed in the rat. Toxicol. Appl. Pharmacol. 34: 264-270, 1975.

Table 1

In Vitro Covalent Binding of ^{14}C- and ^{3}H-Chloramphenicol (0.1 mM) to Phenobarbital Pretreated Rat Liver Microsomal Proteins Under Various Incubation Conditions

Incubation Conditions	^{3}H-CAP	^{14}C-CAP	Inhibition %
	Covalent Binding (pmoles/mg protein/8 min)		
Complete incubation mixture	413 ± 27	444 ± 24	
- NADPH	83 ± 8	104 ± 20	78
+ N_2	168 ± 12	193 ± 5	58
+ $CO:O_2$ (9:1)	207 ± 15	209 ± 15	52
+ Cysteine (1 mM)	263 ± 25	333 ± 30	30
+ GSH (1 mM)	81 ± 20	88 ± 20	80
+ SKF-525A (0.1 mM)	142 ± 5	160 ± 2	65
+ SKF-525A (1 mM)	10 ± 3	12 ± 3	97

Table 2

In Vitro Covalent Binding of ^{14}C- and ^{3}H-Chloramphenicol (0.1 mM) to Protein in Various Preparations of Liver Tissue from Normal and Phenobarbital (Pb) Pretreated Rats

Tissue Preparation	Pretreatment	^{3}H-CAP	^{14}C-CAP
		Covalent Binding (pmoles/mg protein/20 min)	
Tissue Slices	Normal	7 ± 1	17 ± 3
	Pb	21 ± 4	24 ± 5
Total Homogenate	Normal	8 ± 0	8 ± 0
	Pb	78 ± 12	70 ± 15
10,000 x g Supernatant	Normal	22 ± 2	19 ± 2
	Pb	180 ± 12	146 ± 24
105,000 x g Supernatant	Normal	1 ± 0	1 ± 1
	Pb	12 ± 3	12 ± 3
Microsomes	Normal	69 ± 10	81 ± 11
	Pb	996 ± 164	913 ± 18

Table 3

In Vitro Covalent Binding of ^{14}C-Dichloroacetic Acid (0.1 mM) to Protein in Various Preparation of Liver Tissue from Normal and Phenobarbital (Pb) Pretreated Rats

Tissue Preparation	Pretreatment	^{14}C-Dichloroacetic Acid
		Covalent Binding (pmoles/mg protein/20 min)
Tissue Slices	Normal	15 ± 1
	Pb	15 ± 2
Total Homogenate	Normal	30 ± 3
	Pb	29 ± 3
10,000 x g Supernatant	Normal	18 ± 1
	Pb	14 ± 1
105,000 x g Supernatant	Normal	13 ± 1
	Pb	10 ± 1
Microsomes	Normal	0
	Pb	0

Table 4

Covalent Binding of ^{14}C- and ^{3}H-Chloramphenicol (CAP) to Protein in Various Tissues 24 hours after Intraperitoneal Administration (30 mg/kg) to Normal and Phenobarbital (Pb) Pretreated Rats

Tissue	Pretreatment	^{3}H-CAP	^{14}C-CAP	$\frac{^{14}C\text{-CAP}}{^{3}H\text{-CAP}}$
		Covalent Binding (pmoles/mg protein)		
Liver	Normal	54 ± 12	530 ± 113	10
	Pb	53 ± 8	193 ± 15	4
Kidney	Normal	17 ± 1	110 ± 7	7
	Pb	18 ± 1	83 ± 3	5
Lung	Normal	15 ± 2	105 ± 10	4
	Pb	16 ± 1	71 ± 5	4
Plasma	Normal	8 ± 2	409 ± 31	51
	Pb	14 ± 1	156 ± 5	11
Bone Marrow	Normal	7 ± 2	123 ± 15	18
	Pb	10 ± 1	92 ± 4	9
Precentage of Administered Dose Excreted as ^{14}C-CO_2				
	Normal		4.0 ± 0.4	
	Pb		2.2 ± 0.2	

Table 5

Covalent Binding of ^{14}C-Dichloroacetic Acid to Protein in Various Tissues 24 Hours after Intraperitonial Administration (12 mg/kg) to Normal and Phenobarbital (Pb) Pretreated Rats

Tissue	Pretreatment	^{14}C-Dichloroacetic Acid
		Covalent Binding (Pmoles/mg protein)
Liver	Normal	880 ± 33
	Pb	624 ± 27
Kidney	Normal	357 ± 10
	Pb	240 ± 10
Lung	Normal	287 ± 13
	Pb	252 ± 9
Plasma	Normal	108 ± 45
	Pb	664 ± 33
Bone Marrow	Normal	465 ± 13
	Pb	305 ± 24
Percentage of Administered Dose Excreted as CO_2		
	Normal	29 ± 1
	Pb	15 ± 1

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00830-02 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Role of Cyclic GMP as a Mediator of Secretory Functions of Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Dr. G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP NHLI
	Dr. C. Kapoor	Visiting Fellow	LCP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug-Tissue Interaction

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

SUMMARY OF WORK (200 words or less - underline keywords)

The role of cyclic AMP in pancreatic secretion has not yet clearly been demonstrated. We have studied the role of cyclic GMP in pancreatic enzyme secretions in guinea pig pancreatic slices. We have shown that various agents (caerulein, carbamyl choline, cholocystokinin-octa peptide) that cause increase in pancreatic enzyme secretions do so by increasing intracellular cyclic GMP in the pancreatic acinar cells. This increase in cyclic GMP appears to be mediated by the increase in flux of calcium. The calcium ionophore, A23187, also causes a marked increase in cyclic GMP with a concomitant increase in amylase release. It is also possible to increase amylase secretion by dibutyryl cyclic GMP. From these studies it appears that various hormones and agents cause amylase secretion by increasing cyclic GMP in the cell by increasing Ca^{2+} flux.

PHS-6040
(12-75)

Project Description:

Objectives: Previous studies have implicated cyclic AMP as a mediator of amylase release in pancreas. The experimental evidence for this has not been very convincing. Recently we reported that cyclic GMP is markedly increased in pancreatic acinar in response to various secretagogues cells. Since the cells respond poorly to agents in causing amylase release, we have attempted to correlate the cyclic GMP increase with amylase release in pancreatic slices, with which it has been possible to release almost 80% of tissue amylase with various agents. One of the objectives of this study also has been to show that exogenously added cyclic GMP or its derivatives could mimic hormone actions.

Methods Employed: Male NIH strain guinea pigs weighing 300-350 g were fasted over night and killed by decapitation. The excised pancreas were immersed in Krebs Ringer bicarbonate buffer pH 7.4 containing 1.0 mg/ml of glucose equiliberated with 95% O_2 - 5% CO_2. Pancreatic slices (0.2 mm thick) were cut with sharp scissors and preincubated for 30 min. at 37° C in Krebs-Ringer bicarbonate buffer. At the end of the preincubation, the slices were collected by decantation of medium washed and suspended in fresh medium. Slices, equivalent of 20-30 mg wet weight (2-3 mg protein), were then incubated in 1 ml of Krebs Ringer bicarbonate buffer at 37° C for varying periods of time with various secretagogues. To determine the rate of amylase release and cyclic nucleotide formation in pancreatic slices, 50 ul aliquots of medium were taken at various time periods for the measurement of amylase. Cyclic nucleotides in tissue were extracted by boiling the tissue in buffer in a water bath for 2 min. or by perchloric acid extraction and estimated by a radioimmuno assay procedure of Frandson and Krishna. Amylase released into the medium was assayed according to the method of Rinder Knecht et al. Experiantia 23 805 (1967) using amylase azure blue (Calbiochem) as the substrate. Amylase secretion was expressed as the percent of total amylase released into the medium per 10 minutes.

Major Findings: 1) Carbamyl choline (3×10^{-5} M), caerulein (3×10^{-9} M), cholocytokinin-octapeptide (CCK-OP) (3×10^{-9} M) caused a 10-fold increase in cyclic GMP concentration in pancreatic slices with a few seconds. The concentration reached a maximum within 30 seconds and then fell to a lower value at 2-3 minutes. The cyclic GMP level then increased slowly reaching another plateau value in 10 minutes and remained at this value for up to 30 minutes. The hormone-induced increase in the rate of amylase secretion was about 3-4 fold over the basal rate and rate of amylase release was constant for up to 20 min. Cyclic AMP levels in pancreatic slices did not change significantly during this period.

2) There was a close agreement between the dose response curve of various agents in inducing cellular cyclic GMP accumulation and amylase release. (Table 1)

3) Atropine (10^{-7} M) specifically inhibited the accumulation of cyclic GMP and the increase in amylase release caused by carbamyl choline whereas, it had no effect on cyclic GMP accumulation and enzyme secretion induced by

caerulein or CCK octapeptide (Table 2). Inhibition of cyclic GMP accumulation and amylase release were closely correlated with the ID50 of atropine for both the functions. These results suggest that different receptors are involved for the response of caerulein and CCK-OP even though cyclic GMP is the common intercellular mediator of enzyme secretion.

4) In order to understand the role of Ca^{++} in the amylase secretion, we have examined whether Ca^{++} acts intracellularly to cause enzyme secretion through cyclic GMP. A divalent cation ionophore, A23187, which has been found useful in probing the effect of Ca^{++} in various tissues, has been useful in studying the role of Ca^{++} in increasing cyclic GMP in pancreatic acinar cells. The divalent cation ionophore significantly induced cyclic GMP accumulation within 1-2 min with a concomitant amylase release. Cyclic GMP was increased 3 to 4-fold over the basal within 10 min after addition of A23187 and remained at this level for up to 30 minutes. Atropine did not inhibit the effect of the ionophore. Cyclic AMP levels did not change significantly during this period. Dibutyryl cyclic GMP caused a dose dependent increase in the rate of amylase release in pancreatic slices (ED_{50} 10^{-9} M). This effect was not inhibited by atropine.

Significance to Biomedical Research and the Program of the Institute: The finding that cyclic GMP mediates the amylase secretion in pancreas raises the possibility that similar mechanisms may be involved in other secretory processes including surfactant release from the lung.

Proposed Course of Project: A detailed study of the role of calcium and cyclic GMP in other secretory processes, such as histamine release from mast cells, catecholamine release from adrenal medulla and surfactant release from Type II alveolar epethilial cells will be attempted.

Publications:

1. Krishna, G., Krishnan, N., Fletcher, R.T. and Chader, G.: Effects of light on cyclic GMP metabolism in retinal photoreceptors. J. Neurochemistry. (in press)

2. Chader, Gerald J., Fletcher, R. Theodore, O'Brien, Paul J. and Krishna, Gopal: Differential phosphorylation by GTP and ATP in isolated rod outer segments of the retina. Biochemistry. (in press)

3. Chader, Gerald J., Fletcher, R.T., and Grishna, G.: Light-induced phosphorylation of rod outer segments by guanosine triphosphate. Biochem. & Biophysical Research Comm. 64: 535-538, 1975.

4. Krishnan, N. and Krishna G.: A simple and sensitive assay for guanylate cyclase. Anal. Biochem. 70: 18-31, 1976.

5. Frandsen, E.K. and Krishna, G.: A simple ultrasensitive method for the assay of cyclic AMP and cyclic GMP in tissues. Life Sciences 18: 529-542, 1976.

6. Krishna, G. and Krishnan, N.: A rapid method for the assay of guanylate cyclase. J. Cyclic Nucleotide Res. 1: 293-302, 1975.

7. Christophe, J.P., Frandsen, E.K., Conlon, T.P., Krishna, G. and Gardner, J.D.: Action of cholecystokinin; cholinergic agents and A-23187 on accumulation of cyclic GMP in dispersed guinea pig pancreatic acinar cells. J. Biol. Chem. (in press).

TABLE 1

Agonist concentration required to produce half maximal cGMP accumulation and amylase release in guinea pig pancreatic fragments at 10 min.

Agonist	cGMP Accumulation	Amylase release
Carbamylcholine	1.5×10^{-6} M	1.6×10^{-6} M
Caerulein	3×10^{-10} M	3×10^{-10} M
CCK-OP	3.8×10^{-10} M	3.6×10^{-10} M

TABLE 2

Effect of atropine on carbonyl choline-, caerulin- and CCK-OP-induced cyclic GMP accumulation and amylase release by guinea pig pancreatic fragments

Additions	cGMP (pmol/mg protein/ 10 min)	% Amylase release/ mg protein/10 min
None	0.295	0.58
Atropine 10^{-7} M	0.45	0.85
Carbonyl choline 3×10^{-5} M	3.07	6.2
+ atropine 10^{-7} M	0.31	0.83
Caerulein 3×10^{-9} M	2.20	3.84
+ atropine 10^{-7} M	2.37	4.2
CCK-OP 3×10^{-9} M	2.44	3.7
+ atropine 10^{-7} M	2.48	3.84

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00831-01 LCP

PERIOD COVERED
July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Development of an Animal Model for the Study of Aplastic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP NHLI
	I. Aykac	Visiting Scientist	LCP NHLI
OTHER:	L. Pohl	Guest Worker	LCP NHLI
	B.G. Reddy	Research Investigator	LCP NHLI
	D. Johnson	Chief, Veternary Medicine and Surgery Section, Veternary Research Branch, Division of Research Services	

COOPERATING UNITS (if any)

Veternary Research Branch, Division of Research Resources, NIH

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Section on Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	None

SUMMARY OF WORK (200 words or less - underline keywords)

One of the main aims of this group has been to develop a reliable animal model for the study of drug-induced aplastic anemia. After investigating a number of common laboratory animal models for induction of aplastic anemia by chloramphenical, we concluded that none of them would serve for the specific purpose. Since calves have been used for the study of aplastic anemia induced by feeding trichloroethylene extracted soyabean meal, we have utilized calves for chloramphenicol and trichloroethylene induced aplastic anemia. Even though chloramphenicol produces only a milder form of aplastic anemia compared to trichloroethylene and its glutathione (cysteine) conjugate, namely dichlorovinyl cysteine, which induces a complete bone marrow aplasia, it is possible that by changing certain conditions it may be possible to induce a complete aplasia in bone marrow by chloramphenicol treatment. By using this animal model and its bone marrow cells in cluture we hope to gain a better understanding of the biochemical mechanism involved in durg-induced aplastic anemia.

PHS-6040
(12-75)

Project Description:

Objectives: One of the main objectives of this study has been to follow various stages of aplastic anemia induced by chloramphenicol and trichloroethylene and dichlorovinyl cysteine in calves. Even though it has been known for 60 years that calves are very susceptible to aplastic anemia, it has not been used in the study of aplstic anemia induced by drugs. Since previous studies from this laboratory indicated that chloroform depleted liver glutathione by formation of glutathione conjugate, we investigated the possibility of trichloroethylene might be conjugated *in vivo* with glutathione to form a potent aplastic anemia inducing agents, namely dichlorovinyl glutathione and dichlorovinyl cysteine by comparing the incidence of aplastic anemia in calves after the administration of trichloroethylene and dichlorovinyl cysteine.

Methods Employed: About twenty Holstein calves weighing 55-75 kg of both sexes have been utilized in this study. The calves were fed *ad libitum* cow chow and had free access to water. Blood samples were collected daily for 5 days before the experiment and total blood cell counts including differential count of white blood cells were performed. Chloramphenicol (100 mgs/kg/day) was administered orally in gelatin capsules for 10 days. Trichloroethylene was administered at a dose of 200-300 mgs/kg/day for three days in gelatin capsules. Dichlorovinyl cysteine was administered intravenously at a dose of 1 mg/kg, every 2 hours over a period of 6 hrs (total dose 3 mgs/kg). For each drug treatment an equal number of animals were employed as controls. The duration of the experiment varied with the drugs used in the study, generally chloramphenicol animals were sacrificed after 7 weeks. The animals receiving trichloroethylene were sacrificied after 5-6 weeks and those receiving dichlorovinyl cysteine were killed after three weeks. Whenever possible peripheral blood samples were collected daily except during week ends. Toward the end of the experiment bone marrow biopsies were obtained by sternal puncture and bone marrow smears were fixed immediately with methanol and stained as described below. At the end of the experiments the animals were sacrificed. Necropsies were performed in search of any gross abnormalities of internal organs. Bone marrow from ribs, sternum and femoral head were collected and fixed in buffered formalin or methanol. Sections of liver, lymph nodes, spleen, kidney and thymus and adrenals were fixed, embedded and sectioned. Blood smears were fixed in methanol and stained with Giemsa Wright, May Greenwald giemsa for cellular morphology and brilliant cresol for reticulocyte vital staining. Bone marrow smears were fixed in methanol and stained as above for study of the cytological characteristics of the bone marrow cells. Bone marrow cells were also stained for the presence of free iron. RNA and DNA and their precursors in blood cells were stained by methyl green pyronin staining. In order to differentiate lymphocytes from other leucocytes, histochemical localization of peroxidase was employed. Cytoplasmic vaculization due to oil droplets in bone marrow cells were demonstrated by staining the smears with sudan IV and oil Red O.

Paraffin sections of bone marrow were stained for peroxidase, RNA, DNA, free iron. Sections of other tissues were stained with hemotoxylin and eosin.

Major Findings: Chloramphenicol. On necropsy no gross lesions were noted in the abdominal or thoracic organs except for some hyperemia in sinus membranes. Peripheral blood smears obtained from treated animals stained with various staining techniques revealed a predominance of lymphocytes in comparison with untreated animals. No other morphological changes were noted in red and white cells nor were their numbers altered.

The proerythroblasts in the bone marrow smear and bone marrow sections had vacuoles in the nucleus and cytoplasm. There were also highly localized areas of aplasia in bone marrow sections.

In the kidneys there was dilatation and hyperemia of the vessels in the capular region and glomerular vascular network. Similar dilatation and hyperemia were noted in the blood vessels in the liver. In the lymph nodes there was hyperemia in the capsule with sinus enlargement in the peripheral and trabecular region caused by a proliferation and mobilization of the reticulum cells which resulted in follicular compression and atrophy of the lymph nodes.

Finding of focal aplasia in bone marrow sections is highly indicative of an early stage of aplastic anemia.

Trichloroethylene. On necropsy, hepatomegaly, splenomegaly and hyperemia of the serous membrane lining of the abdominal and thoracic cavities were noted in trichloroethylene-treated animals.

Peripheral blood smears showed the occasional presence of immature blood cells and lymphocytosis were common occurrences. Bone marrow sections and biopsy smears of bone marrow cells stained with special stains revealed areas of partial aplasia and hypoplsia. In partial aplasia, the lymphocytes, reticular cells and a few plasmacytes occupying a smaller area than did the fat cells. The hypoplastic regions contained lymphocytes and erythroblasts but very few granulocyties and arythrocytes. The erythrocytes were distributed in the form of small islands and contained cytoplasmic and nuclear vacuoles. These signs are characteristic of aplastic anemia in humans.

The examination of spleen sections revealed hyperemia, vasodilation of the vessels, and the trabecules, hypoplasia of the red pulp and atrophy of the white pulp. The sections of lymph nodes showed some vasodilation and thickening of the vessels and atrophy of secondary follecules. One of the characteristic findings in the lymph nodes was the dilation of the sinuses. Hyperemia was also seen in the kidney, liver and thymus. In the kidney, the glomerulas was dilated in the area of Bowmans capsule and erythrocytes were present in the tubular region. The liver showed hyperemia and vasodilation of the central lobular vein filled with erythrocytes surrounded by lymphocytes.

Dichlorovinyl cysteine. The body temperature remained normal until the 18th day of treatment, but elevated rapidly to 107°F on the 19th day and stayed at the same temperature for 2 more days. On necropsy, hyperemia and vasodilation were noted in the heart, lung and serous membranes. Extensive hemorrhage was seen in the kidneys and slight moderate amount of hemorrhage were noted in the lymph nodes, thymus, adrenals and spleen. Red bone marrow was fatty, jaundiced and had a pink color with a gelatinous consistancy. Macroscopic examination of the peripheral blood smears showed lymphocytosis, leucopaenia and thrombocytopenia. The bone marrow smears and sections revealed characteristic picture of complete aplasia. There was seldom the presence of any types of cells, occasionally pockets of lymphocytes were scattered among the reticulum cells. There was a moderate amount of necrosis among the fibrous tissue. Characteristic changes of aplastic anemia were also seen in the spleen, lymph nodes. Extensive dilation and hemorrhage were seen under the capsule and trabecules of the spleen. The red pulp of the spleen was enlarged and filled with proliferating reticulum cells and reticulocytes. The lymph node sinuses were enlarged and invaded by proliferating reticulum cells which resulted in atrophy and sometimes complete disappearance of secondary folicules. Hemorrage and vasodilatation were also seen in the heart, liver, lung, thymus and brain. This may be due to complete absence or marked reduction of platelets.

These results clearly indicate the possibility of induction of aplastic anemia in calves to varying degrees with various agents. Even though the aplastic anemia induced by chloramphenicol is milder than that produced by dichlorovinylcysteine, it appears that calves may serve as an animal model for the study of the mechanism of chloramphenicol-induced aplastic anemia.

Significance to Biomedical Research and to the Program of the Institute: The finding that calves could serve as an animal model for drug-induced aplastic anemia should greatly help in the understanding of the mechanism of drug-induced aplastic anemia.

Proposed Course of Project: The mechanism by which chloramphenicol and dichlorovinyl cysteine will be investigated both *in vivo* and *in vitro* using bone marrow cells and labeled drugs. We shall try to correlate the capacity of covalent binding of various analogs of these compounds to their capacity to produce aplastic anemia.

Publications:

Reddy, B.G., Pohl, L.P. and Krishna, G.: The requirement of the gut flora in nitrobenzene-induced methemoglobinemia in rats. Biochem. Pharmacol. In press.

Rao, G.S. and Krishna G.: Drug-nitrite interactions: Formation of N-nitroso, C-nitroso and nitro compounds from sodium nitrite and various drugs under physiological conditions. J. Pharm. Sci. 64: 1579-1581, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00832-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The role of cyclic GMP as a possible mediator of platelet aggregation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Dr. G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP NHLI
	Dr. E. Agradi	Visiting Fellow	LCP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug-Tissue Interaction

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

Cyclic AMP has been shown to mediate prostaglandin-induced disaggregation of blood platelets. The role of cyclic GMP in platelet aggregation is not clear. The role of cyclic GMP in blood platelet aggregation was investigated by showing arachidonic acid caused an activation of guanylate cyclase in a cell free system. This activation of the enzyme was completely inhibited by incubation under N_2 and by indomethacin which inhibited the cyclo-oxygenase, the enzyme responsible for this conversion of arachidonic acid to thromboxane and various prostaglandin intermediates. These results indicated that arachidonic acid, which is released by various aggregating agents, could be converted into an intermediate which activates guanylate cyclase and thus could increase cyclic GMP. This increase then could mediate the aggregation of blood platelets.

PHS-6040
(12-75)

Project No. Z01 HL 00832-01 LCP

Project Description:

Objectives: Cyclic AMP has been implicated as a mediator of prostaglandin in inhibiting platelet aggregation. However, the role of cyclic GMP in platelet aggregation has not been clearly understood. Recent studies indicate that thomboxane, a by/product formed during prostaglandin synthesis from arachidonic acid may be responsible for the aggregation of platelets induced by various agents. Various attempts to implicate cyclic GMP as a mediator of aggregation in platelets have not been very successful. Since we have shown previously that guanylate cyclase is present in platelets, we have examined the possibility of whether the guanylate cyclase can be activated either by arachidonic acid, which is released by aggregating agents, or by one of its intermediates formed during prostaglandin synthesis.

Methods Employed: Platelets were isolated from human blood using standard procedures. Platelets were homogenized in Tris-HCl buffer and guanylate cyclase was assayed in various fractions obtained by differential centrifugation.

Major Findings: Arachidonic acid caused a dose dependent activation of guanylate cyclase associated with both the soluble and the membrane fractions of platelets. Half maximal activation was obtained with 5-10 uM arachidonic acid. This activation was time dependent and required oxygen and was completely inhibited under nitrogen. Indomethacin, a specific inhibitor of cyclo-oxygenase involved in prostaglandin and thromoxane synthesis, inhibited arachidonic acid activation of guanylate cyclase.

The effect of endogenously released arachidonic acid from platelet phospholipides by the action of phospholipase A_2 in the activation of guanylate cyclase was examined. Phospholipase A_2 caused a marked activation of guanylate cyclase (3-fold) which was inhibited by incubation under nitrogen. These results indicated that activation of guanylate cyclase by arachidonic acid may be due to a metabolite. This metabolite appears to be formed during prostaglandin synthesis, since the activation of guanylate cyclase and arachidonic acid was specifically inhibited by indomethacin and required oxygen for its formation.

Significance to Biomedical Research and the Program of the Institute: The finding that cyclic GMP may be involved in the arachidonic acid induced blood platelet aggregation may be helpful in the understanding and solving problems involving thrombosis.

Proposed Course of Project: Since these results indicate that guanylate cyclase in platelets is activated by arachidonic acid, its effect on the cyclic GMP level in the intact platelets will be studied. Attempts will be made to isolate and characterize the intermediates involved in the activation fo guanylate cyclase by arachidonic acid using ^{14}C labeled arachidonic acid both in cell free systems as well as in the intact cells.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00833-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The role of cyclic cytidine monophosphate (CMP) in malignant cell growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	D.G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP NHLI
	D.R. Gaion	Visiting Fellow	LCP NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Tissue Interactions

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

Cyclic cytidine 3', 5' monophosphate (cyclic CMP) has been implicated in cell growth. Cytidylate cyclase, the enzyme responsible for the synthesis of cyclic CMP was not detected in both fetal and regenerating liver even with the use of a very highly sensitive method for the assay of the enzyme. However, the enzyme was detected in the leukemia cells (L1210) grown either in tissue culture or in the intraperitonial cavity of mice. The level of enzyme activity was however very small. Since it was not possible to raise antibody against cyclic CMP, it has not been possible to assay the presence of cyclic CMP in liver or leukemia cell culture. It is possible that cyclic CMP may be involved in malignant cell growth but more experiments are necessary to implicate cell growth with increase in cylic CMP.

PHS-6040
(12-75)

Project Description:

Objectives: Cyclic AMP has been implicated as an inhibitor of cell growth in certain firbroblast cultures. Recently it has been proposed that a new cyclicnucleotide namely, cyclic 3', 5' cytidine monophosphate (cyclic CMP) may be involved in malignant growth. Since there are no reliable methods for the assay of cyclic CMP, our objective has been to develop a simple and sensitive assay for the enzyme that catalyzes the synthesis of cyclic CMP, and to examine the role of cyclic CMP in cell growth both malignant and non-malignant.

Methods Employed: Several tissues were assayed: 1) Fetal liver from 1 day old rats.2) The regenerated liver from 1-5 days after partial hepatactomy of mice and rats 3) Leukemia cells (L1210) grown in mice and tissue culture.

Cytidylate cyclase was analyzed according to the method developed in the laboratory. This involved incubation of the tissue homogenates in a final volume of 100 ul with [α-^{32}P] CTP in the presence of 3 mM Mn++ and 1 mM [^{3}H] cyclic CMP and 0.1 mM isobutyl methyl xanthine as a phosphodisterase inhibitor in 40 mM Tris HCl buffer (pH 7.4). The reactions were terminated by addition of EDTA. The incubation mixtures were chromatographed on Dowex 50 followed by alumina columns. This method can detect the formation of as little as 0.1 pmole of cyclic CMP/min.

Major Findings: Even with the utilization of a very sensitive method we have not been able to detect cytidylate cyclase in fetal liver or regenerating livers of both mice and rats. In the leukemic cells we have sometimes detected trace activity which was only 2 to 3 times the value obtained with denatured enzyme. Our inability to detect enzyme activity was not due to degradation of cyclic CMP during the assay since we employed [^{3}H] cyclic CMP during the assay so that we can follow its degradation and the recovery has ranged from 80-90%. We have examined various parameters including various divalent ion requirements and at various substrate and protein concentrations. So far we have not detected any appreciable activity.

Preliminary attempts to make antibodies against cyclic CMP have failed. Thus it has not been possible to determine whether cyclic CMP is increased during cell growth utilizing a radioimmuno assay. However, we attempted to prelabel CTP in the cell with [^{32}P]phosphate. The [^{32}P]cyclic CMP formation was very low and could not be reliably measured.

Significance to Biomedical Research and to the Interest of the Institute: Even though the direct experimental evidence is lacking it is still possible that CMP may occur in cells and may be responsible for growth especially in malignant growths. If further studies are successful in demonstrating cyclic CMP presence during malignant growth, it may be highly useful in diagnosing malignant growth at an early stage.

Proposed Course of Project: A specific radioimmunoassay for cyclic CMP will be developed which would enable us to test the possibility that cyclic CMP is involved in malignant tissue growth.

Publications: NONE.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00834-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Study on the role of cyclic nucleotides as mediators of hormone functions in cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Dr. G. Krishna	Chief, Section on Drug-Tissue Interactions	LCP NHLI
	Dr. R. Gaion	Visiting Fellow	LCP NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interactions

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

The role of cyclic AMP in hormone-induced lipolysis in fat cells has been well established. However the role of cyclic GMP in hormone-induced lipolysis is not clear. We have studied the effect of various hormones on cyclic GMP as well as cyclic AMP levels in isolated fat cells. Epinephrine and acetyl choline causes a marked increase in cyclic GMP in fat cells within seconds while cyclic AMP level was increased by epinephrine more slowly. The early increase of cyclic GMP was potentiated by Propranolol, a beta adrenergic blocking agent while the later increase in cyclic GMP was blocked by phentolamine, an alfa adrenergic blocking agent indicating that an adrenergic mechanism may be involved in cyclic GMP increase in the fat cell. The role of cyclic GMP in hormone-induced lipolysis is not yet clearly understood. It may have a modulatory role on hormone-induced lipolysis.

Project Description:

Objectives: Cyclic AMP as the mediator of hormone-induced lipolysis in fat cells has been well documented. However, the role of cyclic GMP in these cells are not clear. It has been reported recently that cyclic GMP amy mediate the inhibitory effect of insulin on lipolysis but the experimental evidence has not been very convincing. One of the main problems has been the difficulty involved in measuring cyclic GMP which occurs in very small amounts in fat cells. This has been over come by the development of a very simple radioimmuno assay of cyclic GMP in this laboratory. The main objective of this study is to examine the role of cyclic GMP in mediating or modulating various hormone actions in fat cells.

Methods Employed: Isolated fat cells from male Sprague Dawley rats weighing 150 g epididymal fat pads were utilized in the study. Cyclic AMP, and cyclic GMP were converted to their succinate derivatives which were measured by radioimmuno assay. Lipolysis was measured by the rate of glycerol release from fat cells in response to hormones.

Major Findings: Epinephrine (10^{-8} to 10^{-6} M) induced a marked increase (six-fold) in cyclic GMP levels in fat cells within seconds reaching a peak at 15-30 seconds. The cyclic GMP level then declined to almost basal value within 1-2 min and then it started to rise again, reaching a plateau in about 6-8 minutes. The dose of epinephrine to induce the maximal response in 30 seconds was much lower [($< 10^{-8}$ M), higher doses were inhibitory] than the dose required to produce the maximal response in 6 min (10^{-6} M), thus indicating these responses may not be related. Epinephrine also induced a dose-dependent increase in cyclic AMP in fat cells (10^{-6} M for maximal response) but the rise in cyclic AMP was slower and reached a plateau in about 6 minutes. It seems likely that there was a strict correlation between this response and lipolysis. The early increase in cyclic GMP by epinephrine was mediated through an alpha adrenergic receptor because the early effect was potentiated by a beta blocking agent, propranolol, while the later effect was inhibited by an alpha blocking agent. Thus it seems probable that cyclic GMP mediates the alpha adrenergic function in fat cells but further experiments are needed to confirm this view. Acetylcholine also caused a marked increase in cyclic GMP levels in fat cells within seconds reaching a peak in about 30 seconds. The cholinergic stimulation of fat tissue *in vivo* causes a reduction in lipolysis caused by adrenergic stimulation and this modulation may be mediated through cyclic GMP. Further experiments to clarify the role of cholinergic and alpha adrenergic function in fat cells are in progress.

Significance to Biomedical Research and to the Program of the Institute: The finding that alpha adrenergic stimulation may be mediated through a rise in cyclic GMP raises an interesting possibility that alpha adrenergic stimulation in blood vessels which causes contraction of the smooth muscle, may be mediated through cyclic GMP. This could have some implications in the understanding of the mechanism and treatment of hypertension.

Proposed Course of Project: The role of cyclic GMP in mediating cholinergic and adrenergic functions will be studied in detail including in reference

to the role of Ca^{++} as a possible mediator in the activation of guanylate cyclase in the cell. These studies will be extended to other systems such as blood vessels and lung.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00851-03 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Paraquat Toxicity in Rat and Mouse

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. M. Maling	Chief, Section on Physiology	LCP NHLI
OTHER:	E. A. B. Brown	Pharmacologist	HE NHLI
	J. R. Gillette	Chief, Laboratory of Chemical Pharmacology	LCP NHLI
	J. H. Ware	Statistician, Biometrics Research Branch, Division of Heart and Vascular Diseases	NHLI

COOPERATING UNITS (if any)

Hypertension-Endocrine Branch, NHLI
Biometrics Research Branch, Division of Heart and Vascular Diseases, NHLI

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Section on Physiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	1.2	2.0

SUMMARY OF WORK (200 words or less - underline keywords)

This project is a study of mechanisms involved in the pulmonary toxicity of paraquat, an herbicide extensively used in agriculture. In rats, the 48-hr mortality from paraquat ip was increased by sc injection of a single dose of a beta adrenergic agonist (l-isoproterenol, salbutamol, carbuterol, or fenoterol). Pretreatment with dl-propranolol, l-propranolol, or bunolol, all beta blocking agents, completely blocked isoproterenol-induced potentiation of paraquat toxicity. Other beta blocking agents and d-propranolol were less or not effective. Isoproterenol-induced potentiation of paraquat toxicity and its blockade by propranolol were also demonstrated in mice. ^{14}C-labeled paraquat, N-methylnicotinamide (NMN), or p-aminohippuric acid (PAH) was injected into the tail vein of rats and plasma disappearance of radioactivity was followed in blood from the retro-orbital sinus. Since isoproterenol (0.3 mg/kg), injected sc at the same time as the test compound, reduced the clearance of both basic (paraquat and NMN) and acidic (PAH) compounds, it must have reduced renal blood flow. The reduced clearance contributed at least partially to the increased toxicity of paraquat. There was no clear relationship between the effect of a drug on paraquat mortality and its effect on paraquat uptake by lung slices in vitro. We have failed to detect, in vitro or in vivo, production of the superoxide free radical by paraquat.

PHS-6040
(12-75)

Project Description:

Objectives: During 1975-1976, experiments have been performed with the following objectives: 1) To obtain evidence for a beta adrenergic receptor component of paraquat toxicity. 2) To determine the effect of isoproterenol on paraquat clearance. The reduction of paraquat clearance by isoproterenol explains at least partially the isoproterenol-induced potentiation of paraquat toxicity. 3) To compare the inhibition by a drug of paraquat uptake by lung slices *in vitro* with the drug's effect on paraquat toxicity *in vivo*. 4) To obtain evidence for or against the involvement of the superoxide free radical in paraquat toxicity.

Methods Employed: Plasma levels of radioactivity have been measured on blood samples obtained from the retro-orbital sinus at appropriate intervals after injection into a rat's tail vein of ^{14}C-labeled paraquat, N-methylnicotinamide (NMN), or p-aminohippuric acid (PAH). Clearance values for paraquat, NMN and PAH have been calculated from the relationship, Cl = Dose/AUC, in which AUC is the area under the curve of plasma disappearance graphs.

Major Findings: In rats, the 48-hr mortality from paraquat was markedly increased by s.c. administration of a single dose of a beta adrenergic agonist (l-isoproterenol, salbutamol, carbuterol, fenoterol). Pretreatment with dl-propranolol (25 mg/kg s.c. 15 min before injection of paraquat i.p.) completely blocked the beta agonist-induced potentiation of paraquat toxicity. Isoproterenol-induced potentiation of paraquat toxicity and its blockade by dl-propranolol were also demonstrated in mice. In rats, bunolol and l-propranolol also completely blocked isoproterenol-induced potentiation of paraquat toxicity. Less effective were d-propranolol, Kö 592 (1-[3-methylphenoxy]-3-isopropylaminopropanol hydrochloride), sotalol, or pronethalol. Practolol, butoxamine, methoxamine, or dopamine did not reduce the isoproterenol-induced potentiation of paraquat toxicity. Since practolol, a beta-1 adrenergic receptor blocking agent, did not protect rats against isoproterenol-induced potentiation, it seems likely that the potentiation involves beta-2 receptors. This hypothesis is supported by the fact that three beta-2 agonists (salbutamol, carbuterol, and fenoterol) all potentiated paraquat toxicity. Although some investigators have classified butoxamine and methoxamine as beta-2 blocking agents, these drugs failed to protect rats against isoproterenol-induced potentiation of paraquat toxicity. The classification of methoxamine and butoxamine is controversial. Our experiments do not support their classification as beta-2 blocking agents.

Isoproterenol reduced the clearance of paraquat by about 50%, as calculated from plasma disappearance curves. Since isoproterenol reduced the clearance of both basic (paraquat and NMN) and acidic (PAH) compounds, it probably reduced renal blood flow. Dopamine was tested for its effect on isoproterenol-induced potentiation because it increases renal blood flow. Its failure to block isoproterenol-induced potentiation suggests that the interactions among these drugs is complicated and that other mechanisms in addition to clearance effects may be involved.

There was no correlation between the ability of a drug to inhibit uptake of paraquat by rat lung slices and its effect on 48-hr paraquat mortality. Dopamine was the most effective inhibitor of paraquat uptake tested, with an approximate IC50 (concentration which inhibits 50% of paraquat uptake) of 6×10^{-5}M. Yet treatment with dopamine (0.3 and 3.0 mg/kg s.c.) did not reduce paraquat mortality in otherwise untreated rats or in isoproterenol-potentiated rats. Among the beta blocking agents, the most effective in blocking paraquat uptake were dl-propranolol and l-propranolol, which were approximately equally effective in blocking uptake in vitro, in protecting rats against isoproterenol-potentiated paraquat toxicity, and in reducing paraquat mortality in otherwise untreated rats. However, these beta blocking agents were no more effective than imipramine, desipramine, or histamine and were less effective than SKF 525-A or l-norepinephrine in blocking uptake of paraquat by rat lung slices. Despite their effectiveness in inhibiting uptake of paraquat, none of these compounds protected rats against paraquat toxicity. The beta blocking agents were the only class of drugs tested which showed a trend towards reducing the 48-hr mortality from paraquat. In vitro, many of these beta adrenergic blocking compounds inhibited paraquat uptake by lung slices, but the concentrations required were relatively high and ranking the compounds according to their ability to inhibit uptake gave a different order from ranking by their effects on isoproterenol-induced potentiation or paraquat mortality or their effect on paraquat mortality alone.

In our report for 1974-1975, we described our failures to "detect a paraquat-induced increase in diene conjugation of lung microsomal lipid, either from lungs of rats injected with paraquat i.p. or from lung slices incubated in vitro with high concentrations of paraquat, either in air or 95% O_2, 5% CO_2". We also failed to detect paraquat-induced increases in thio-barbituric acid (TBA) reactants. During 1975-1976 we conducted several experiments designed to detect formation of superoxide free radicals and peroxides. One experiment involved the incubation of rat lung slices with paraquat (10^{-3}M) and NaCN (10^{-3}M). Since NaCN inhibits superoxide dismutase (Fridovich, Advances in Enzymology, 41: 35, 1974), incubation in the presence of NaCN should permit the accumulation of superoxide free radicals, peroxides, and TBA reactants, such as malondialdihyde. No TBA reactants were detected, despite the presence of NaCN.

Significance to Biomedical Research and the Program of the Institute: A study of the pulmonary toxicity induced by paraquat in rodents may give insight into mechanisms leading to pulmonary fibrosis in humans. Studies of the effects of drugs on paraquat poisoning in mice and rats may suggest better treatments for paraquat poisoning in man. The experiments on isoproterenol-induced potentiation of paraquat toxicity suggest possible dangers in the use of beta adrenergic agonists in the treatment of paraquat poisoning.

Proposed Course of Project: Experiments are now in progress to determine the effect of paraquat on the covalent binding of ^{3}H-dl-epinephrine to lung and liver slices and homogenates, incubated in vitro. In another project in this laboratory (Dybing et al., No. Z01 HL 00801-01 LCP), the covalent binding of α-methyldopa and other catechols to microsomal protein has been shown to

to be mediated through the superoxide anion, since the binding is inhibited by superoxide dismutase. The covalent binding of epinephrine to protein can be used therefore as a sensitive method of detecting the formation of the superoxide free radical.

Other methods of producing pulmonary edema in rats will be studied.

Appendix: A contract (PD-364077-6) has been arranged with Dr. William J. Waddell, Prof. of Pharmacology, University of Kentucky College of Medicine (Lexington, Kentucky 40506) to produce frozen tissue autoradiographs of the distribution of ^{14}C-paraquat in mice, with the objective of determining whether paraquat is localized within specific cells in the lung.

Publications:

Maling, H. M., Stripp, B., Sipes, I.G., Highman, B., Saul, W. and Williams, M.A.: Enhanced hepatotoxicity of carbon tetrachloride, thioacetamide, and dimethylnitrosamine by pretreatment of rats with ethanol and some comparisons with potentiation by isopropanol. Toxicol. Appl. Pharmacol. 33: 291-308, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00877-03 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Hydrazines 2. Assays for mono- and diacetylhydrazine in human plasma and urine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	S.D. Nelson	Staff Fellow	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

Two major metabolites in man of the widely used antituberculosis drug, isoniazid, are monoacetylhydrazine and diacetylhydrazine. Because either one or both of these hydrazines may be responsible for isoniazid liver injury, we are developing procedures to monitor these two metabolites in human plasma and urine. Gas chromatographic assays have been developed for both monoacetylhydrazine and diacetylhydrazine with sensitivities for plasma assays ranging down to 100 ng/ml. The assay for diacetylhydrazine is erratic, however, and a more specific and accurate assay for both mono- and diacetylhydrazine is being developed using stable isotope internal standards and combined gas chromatography-mass spectrometry. Once conditions are worked out we will minitor the effects of inducers and inhibitors of drug metabolism on the disposition in vivo of these potentially-toxic metabolites. Such studies offer the possibility that therapeutic regimens can be developed which decrease either the production of such metabolites or their subsequent biotransformation to reactive metabolites.

PHS-6040
(12-75)

Project Description:

Objectives: A number of recent clinical and biocheical studies from laboratory have produced evidence that the hydrazine moiety of the widely used antituberculosis drug, isoniazid, is responsible for the serious hepatitis associated with this drug. Two major metabolites arise from this moiety, monoacetylhydrazine (AcHz) and diacetylhydrazine (DiAcHz). In order to monitor the formation of these potentially toxic metabolites in man, we are developing assays using gas chromatography and combined gas chromatography-mass spectrometry.

Methods Employed: For the GC assay for plasma levels of AcHz, a Perkin Elmer Model 900 gas chromatography equipped with a flame ionization detector and a 6 food glass column (2 mm id) containing 10% OV-17 on Gas Chrom-Q 100/120 mesh, was used. Both injector and detector temperatures were held at 300° while the column was maintained isothermally at 250° with a nitrogen flow rate of 30 ml/min.

Blood samples were drawn from one healthy normal volunteer, and centrifuged for 20 min at 15,000 rpm to separate blood cells from plasma. Five ml plasma aliquots were withdrawn and the pH was immediately adjusted to 3.0 using 1.0 N HCl. After the addition of p-dimethylaminobenzaldehyde (Eastman), 0.1 ml of a 20 mg/ml solution in 1.0 N HCl, and gentle mixing, samples were allowed to incubate at room temperature for 60 min. Addition of methylene chloride to each sample (2X30 ml) was followed by gentle aggitation and centrifugation, and the combined organic layers were dried over anhydrous magnesium sulfate before being rotary evaporated. The resultant residues were reconstituted in 0.1 ml ethyl acetate and 2.0 microliters were used for injection. A retention time of 4.4 min was observed for the derivative of acetylhydrazine and repeated plasma blanks failed to reveal the presence of interfering peaks. Standard curves determined from both water and plasma showed comparable linear responses to acetylhydrazine concentrations ranging between 0.05 micrograms/ml and 100 micrograms/ml using peak heights as a measure of the amount of hydrazone present.

Attempts to develop a GLC method for the determination of N^1,N^2-Diacetylhydrazine (DiAcHz) in water and urine: After a constant amount of iproniazid (internal standard) and varying amounts of diacetylhydrazine were added to 25 ml aliquots of water or human urine, the solutions were freeze-dried and the residues were extracted with methanol (2X25 ml) followed by filtration. The methanol extracts were combined, their volumes reduced by rotary evaporation, and the concentrated solutions were applied to 1000 micron avicel TLC plates (Analtech) and the chromatograph developed using n-butanol:ethanol:0.4 M NH_4OH (4:1:1). DiAcHz (r.f. - 0.71) was scraped from the plate, eluted with methanol, and the solution was rotary evaporated to dryness. Addition of 0.5 ml Bis-trimethylsilylacetamide (Pierce) was followed by heating for 30 min at 60°C and an injection of 2.0 microliters onto the GC column described earlier. The column temperature was held constant at 90°C (N_2 flow rate - 20 ml/m-n) while the injector and detector temperatures were maintained at 200°C. Retention times of 6.5 and 11.2 min

were observed for the silylated derivatives of DiAcHz and iproniazid, respectively. A standard curve, constructed by plotting the ratios of DiAcHz to iproniazid heights vs DiAcHz concentration was linear for aqueous s lutions, but was quite variable for urinary levels. Attempts to improve the reliability of this analytical procedure involved the use of 3% OV-17, 3% OV-1, 3% SE-30, 1% CHROM-103, and 3% XE-60 GLC columns with and without the prior use of DEAE of XAD-2 column chromatography, but none of the combinations tested to date has been effective in accomplishing this goal.

Major Findings: Initial experiments with 4 patients receiving 300 mg oral doses of isoniazid for positive tuberculin tests show that concentrations of AcHz in venous blood samples, measured as its p-dimethylaminobenzaldehyde hydrazone by the GC assay described in Methods, range from 100 ng/ml to 500 ng/ml.

Attempted assay of DiAcHz as a sildyl derivative showed erratic results due to nonlinearity in the GC measurements.

Significance to Biomedical Research and to the Program of the Institute: Since certain patients appear to be at high risk when receiving isoniazid therapy, a method for monitoring potentially toxic metabolites should prove useful. Even more attractive would be the use of such an assay to monitor the effects of inducers and inhibitors of drug metabolism on the in vivo disposition of these potentially toxic metabolites. Such studies of the modulation of isoniazid metabolism offer the possibility that therapeutic regimens can be derived which decrease either the production of such toxic metabolites or their subsequent biotransformation of reactive acylation species but without compromising the therapeutic actions of the drug.

Proposed Course of Project: A more specific, and accurate assay for both AcHz and especially DiAcHz will be developed using stable isotope standards (d_3-AcHz and d_6-DiAcHz). These standards will be immediately added in known amounts to plasma or urine samples from patients receiving INH and the described assays will be performed. However, the GC will be coupled to a mass spectrometer (V.G. Micromass 16F) so that only the particular parent ions in question will be monitored thereby assuming a specific assay for the compounds in question. The use of the stable isotope standards eliminates problems of erratic recoveries and GC response, particularly in the case of DiAcHz.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00880-02 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Hydrazines 1. Necrosis and covalent binding of mono- and diacetylhydrazine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	S.D. Nelson	Staff Fellow	LCP NHLI
Other:	W.R. Snodgrass	Guest Worker	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS and Dr. Snodgrass holds an NIH Research Fellowship in NHLI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

~~Clinical Pharmacology~~

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

Both monoacetylhydrazine and diacetylhydrazine are metabolites of isoniazid in man and rat. Monoacetylhydrazine has previously been shown to be a potent hepatotoxin in rats, and, based on several studies, oxidation of this compound by microsomal P-450 enzymes, appears to cause the serious hepatitis observed in some patients receiving isoniazid therapy. However, diacetylhydrazine, which is formed by acetylation of monoacetylhydrazine, is found in human and rat urine samples in greater amounts. Therefore, in experiments using both monoacetylhydrazine and diacetylhydrazine, we tested for hepatotoxicity and covalent binding of these metabolites in rats. As determined previously, monoacetylhydrazine causes liver necrosis in phenobarbital pretreated rats and covalently binds to hepatic tissue both in vivo and in vitro. In contrast, diacetylhydrazine did not produce liver lesions and did not significantly bind to hepatic tissue in vivo or in vitro. Potential chronic hepatotoxicity in rats of both monoacetylhydrazine and diacetylhydrazine is now being studied.

PHS-6040
(12-75)

Project Description:

Objectives: Both monoacetylhydrazine and diacetylhydrazine are metabolites of isoniazid in man and rat. Monoacetylhydrazine has previously been shown to be a potent hepatotoxin in rats, and, based on several studies, oxidation of this compound by microsomal P-450 enzymes, appears to cause the serious hepatitis observed in some patients receiving isoniazid therapy. However, diacetylhydrazine, which is formed by acetylation of acetylhydrazine, is found in human and rat urine samples in greater amounts. Therefore, we tested this compound for its potential hepatotoxicity and potential for covalent binding to hepatic tissue in vivo and in vitro.

Methods Employed: Fischer rats were pretreated with phenobarbital for 4 days (75 mg/kg, ip). Studies in vivo were carried out by ip administration (100 mg/kg) of either monoacetylhydrazine or diacetylhydrazine to phenobarbital pretreated rats. Twenty-four hr later liver tissue was removed and fixed in buffered formalin solution. Tissues were then stained (H&E) and mounted by EPL Laboratories and the slides read for cell necrosis.

Six animals from the same pretreatment group were administered radiolabeled (^{14}C) monoacetylhydrazine (100 mg/kg); to another 6 animals was administered radiolabeled (^{3}H) diacetylhydrazine (100 mg/kg). After 6 hours, samples of liver, kidney, and muscle tissue were removed and the covalent binding of radiolabel to the tissue was determined.

Liver microsomes were isolated from an additional 3 animals in the phenobarbital pretreatment group. Covalent binding of radiolabeled monoacetylhydrazine and diacetylhydrazine was determined by previously described methods.

Major Findings: As determined previously, monoacetylhydrazine causes liver necrosis in phenobarbital pretreated rats and covalently binds to hepatic tissue both in vivo and in vitro (Table 1). In contrast, diacetylhydrazine did not lead to the hepatic tissue liver necrosis and did not significantly bind to hepatic tissue in vivo or in vitro (Table 1).

Significance to Biomedical Research and to the Program of the Institute: These results provide further evidence that acetylhydrazine is the proximate reactive metabolite of isoniazid leading to hepatic necrosis. Diacetylhydrazine appears to be relatively nontoxic at the doses employed.

Proposed Course of Project: Further studies will be carried out in Fischer rats using repeated doses of diacetylhydrazine to determine if hepatic necrosis can be initiated chronically rather than acutely.

Publications:

Black, M., Mitchell, J.R., Zimmerman, H.J., Ishak, K.G., and Epler, G.R.: Isoniazid hepatitis in 114 patients. Gastroenterology 69: 284-302, 1975.

Mitchell, J.R., Long, M.W., Thorgeirsson, U.P. and Jollow, D.J.: Acetylation rates and monthly liver function tests during one year of isoniazid preventive therapy. Chest 68: 181-190, 1975.

Mitchell, J.R., Thorgeirsson, U.P., Black, M., Timbrell, J.A., Snodgrass, W.R., Potter, W.Z., Jollow, D.J. and Keiser, H.R.: Increased incidence of isoniazid hepatitis in rapid acetylators: Possible relation to hydrazine metabolites. Clin. Pharmacol. Ther. 18: 70-79, 1975.

Mitchell, J.R., Nelson, S.D., Snodgrass, W.R. and Timbrell, J.A.: Metabolic activation of hydrazines to highly reactive hepatotoxic intermediates. Proceedings of satellite symposium on Active Intermediates, Sixth International Congress of Pharmacology, Turku, Finland, July 1975.

Mitchell, J.R., Zimmerman, H.J., Ishak, K.G., Thorgeirsson, U.P., Timbrell, J.A., Snodgrass, W.R., Nelson, S.D.: Isoniazid liver injury: Clinical spectrum, pathology and probable pathogenesis. Annals of Internal Medicine 84: 181-192, 1976.

Nelson, S.D., Mitchell, J.R., Timbrell, J.A., Snodgrass, W.R., and Corcoran, G.B.,III: Activation of metabolites of isoniazid and iproniazid to toxic intermediates in man and rat. Science, in press.

Nelson, S.D., Snodgrass, W.R. and Mitchell, J.R.: Chemical reaction mechanisms responsible for the tissue injury caused by monosubstituted hydrazines and their hydrazide drug precursors. Proceedings of the Symposium Workshop on Mutagenesis-Drug Metabolism, in press.

Zimmerman, H.J. and Mitchell, J.R.: Competing risks of tuberculosis and isoniazid hepatitis. Annals Int. Med. June 1976.

Table 1

Hepatic necrosis and covalent binding of acetylhydrazine and diacetylhydrazine in male Fischer rats pretreated with phenobarbital

	Monoacetylhydrazine	Diacetylhydrazine
Necrosis	+++	0
Covalent binding *in vivo* (nmoles/mg protein)	0.225 ± .032	0.017 ± .001
Covalent binding *in vitro* (nmoles/mg/15 min)	0.566 ± .040	0.045 ± .006

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00881-02 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Phenacetin-Induced Toxicity: N-Oxidation of Phenacetin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jack A. Hinson	Staff Fellow	LCP-NHLI
OTHER:	J. R. Mitchell	Chief, Section on Clinical Pharmacology	LCP-NHLI

COOPERATING UNITS (if any)

Dr. Hinson was supported by a NIH Research Fellowship 1F22 HL 02206-D1

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms of nephrotoxicity and hepatotoxicity of phenacetin are unknown. Since N-hydroxylation of acetaminophen leads to the production of a hepatotoxic metabolite of this drug, the possible N-hydroxylation of phenacetin was studied. Mass spectral proof has been obtained that phenacetin is N-hydroxylated in vitro. A quantitative assay for the N-hydroxylation of phenacetin has been developed. The N-hydroxylation reaction is catalyzed by a cytochrome P-450 mixed function oxidase, and the rate of the reaction is approximately 10% of the de-ethylation reaction. N-hydroxyphenacetin is further metabolized in the microsomal incubation. An examination of the metabolites showed phenacetin was a major metabolite and another metabolite water soluble, presumably a deacetylated form. Both reactions required NADPH.

PHS-6040

Project Description:

Objectives: Previous studies have shown that a toxic metabolite of acetaminophen is formed during metabolism of the drug in the liver by a cytochrome P-450-dependent mixed-function oxidase. Evidence has been presented that this toxic reactive metabolite is N-acetyl-4-benzoimidoquinone which arises through N-hydroxylation of the parent drug followed by non-enzymatic rearrangement. Phenacetin also causes centrilobular necrosis in hamsters. Since N-hydroxyphenacetin will spontaneously rearrange to N-acetyl-4-benzoimidoquinone, a known arylating agent, we examined the capacity of hepatic microsomes to N-hydroxylate phenacetin.

Methods Employed: N-Hydroxyphenacetin was synthesized from p-nitrophenatol following a modification of previously described techniques. ^{14}C-Acetyl chloride was used to synthesize the radiolabeled compound. All other procedures were standard.

Major Findings: A metabolite with the chromatographic characteristics of N-hydroxyphenacetin was isolated from an incubation containing ^{3}H-phenacetin and hamster liver microsomes. Mass spectrometry showed the metabolite to be N-hydroxyphenacetin.

A quantitative assay for N-hydroxyphenacetin has been developed and its specificity established by crystallization of the metabolite with ynthetic unlabeled N-hydroxyphenacetin. The rate of the N-hydroxylation reaction was approximately 10% of the de-ethylation reaction. The reaction was dependent upon NADP and molecular oxygen and was inhibited by carbon monoxide indicating that it was catalyzed by cytochrome P-450. Sodium fluoride increased the rate of N-hydroxylation.

N-hydroxyphenacetin was further metabolized in the microsomal incubation media. An examination of the metabolites showed that phenacetin was a major metabolite. Another metabolite was water soluble and was presumed to be a deacetylated form. Both reactions required NADPH.

Significance to Biomedical Research and to the Program of the Institute: The present studies demonstrate phenacetin is N-hydroxylated *in vitro* and is a significant metabolite.

Proposed Course of Project: Since N-hydroxyphenacetin is an important *in vitro* metabolite, the potential hepatoxicity and nephrotoxicity of this metabolite will be studied.

Publications:

Hinson, J.A., Mitchell, J.A. and Jollow, D.J.: Studies on the toxicity of acetanilide derivatives: Evidence for the microsomal N-hydroxylation of p-chloroacetanilide. Molecular Pharmacology 11: 462-469, 1975.

Project No. _____

Hinson, J.A., Mitchell, J.R. and Jollow, D.J.: N-Hydroxylation of p-chloroacetanilide in hamsters. Biochem. Pharmacol. 25: 599-601, 1976.

Hinson, J.A. and Mitchell, J.R.: N-Hydroxylation of phenacetin by hamster liver microsomes. Drug. Metab. Dispos., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00882-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Metabolic activation of estrogens and 2-hydroxyestrogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	S.D. Nelson	Staff Fellow	LCP NHLI
OTHER:	J.R. Mitchell	Chief, Sect. on Clin. Pharm.	LCP NHLI
	E. Dybing	Fogarty Inter. Fellow	LCP NHLI
	H.A. Sasame	Chemist	LCP NHLI

COOPERATING UNITS (if any)

Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS, Dr. Dybing was a Fogarty International Fellow.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

Various hydroxylated estrogens are major metabolites of estrogens in man and animals, including the 2-hydroxylated catechol estrogens. Our previous work with catechols, such as α-methyldopa, showed that they could be oxidized to electrophilic derivatives which bind to tissue macromolecules. We, therefore, investigated the possibility that estrogens and catechol estrogens might be activated in a similar manner. 2-Hydroxyestradiol, 2-hydroxyestrone and 2-hydroxy-17α-ethynylestradiol were found to be converted by rat liver microsomes to reactive metabolites that become covalently bound to microsomal protein. The covalent binding required microsomes, oxygen, and NADPH. The NADPH could be replaced by xanthine-xanthine oxidase which is known to generate superoxide anions. The binding was substantially inhibited by superoxide dismutase, 30% in those incubations containing NADPH and 98% in those containing the xanthine-xanthine oxidase system. The binding was inhibited 70% in an atmosphere of $CO:O_2$ (9:1). These experiments plus other studies using various inhibitors and inducers of the P-450 system, are consistent with a mechanism for the covalent binding of estrogens and 2-hydroxyestrogens to microsomes that requires oxidation of the catechol nucleus by cytochrome P-450-generated superoxide anion. Studies are now in progress to determine the significance of these experiments to various kinds of estrogen toxicity which have recently been documented.

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PHS-6040
(12-75)

Project Description:

Objectives: Various hydroxylated estrogens are major metabolites of estrogens in man and animals, including the 2-hydroxylated catechol estrogens. Our previous work with catechols, such as -methyldopa, showed that they could be oxidized to reactive electrophilic derivatives which bind to tissue macromolecules. We have, therefore, investigated the possibility that estrogens and catechol estrogens might be activated in a similar manner.

Methods Employed: Radiolabeled estrogens ([6,7-^{3}H] estradiol, [6,7-^{3}H] estrone, [6,7-^{3}H] -17α-ethinylestradiol) were obtained from Amersham-Searle and recrystallized to constant sp. act. with their unlabeled counterparts purchased from Sigma. Radiolabeled 2-hydroxyestrogens were prepared by published procedures. Livers were obtained from male Fischer rats and microsomes were prepared in an identical fashion from Fischer rats pretreated as follows: 1) phenobarbital (75 mg/kg, i.p. daily for 4 days), 2) cobaltous chloride (30 mg/kg, s.c. twice daily for 2 days), 3) pregnenolone-16 -carbonitrile (75 mg/kg i.p, twice daily for 4 days).

Radiolabeled compounds were incubated with microsomal protein in the presence and absence of various cofactors and other modifiers of the system as described in Table 1. Covalent binding of reactive intermediates to microsomal protein was determined according to previously published methods.

Major Findings: Incubations containing rat liver microsomes, radiolabeled estrogen (estradiol, estrone or 17α-ethinylestradiol) or 2-hydroxyestrogen (2-hydroxyestradiol, 2-hydroxyestrone or 2-hydroxy-17α-ethinylestradiol) and an NADPH-generating system showed substantial irreversible binding of radiolabeled to microsomal protein (Table 1). Omission of the NADPH-generating system almost abolished binding. Addition of superoxide dismutase to the control incubation mixture significantly decreased the irreversible binding of both estrogens and their 2-hydroxylated derivatives by 20 to 35% after subtracting non-NADPH-dependent binding. Replacement of the NADPH-generating system by xanthine-xanthine oxidase, which is known to generate 2-hydroxyestrogens showed high irreversible binding levels that were again markedly reduced by addition of superoxide dismutase.

Pretreatment of animals with phenobarbital had no effect either on the irreversible binding of 2-hydroxyestradiol to microsomal protein or on the inhibition of the binding by superoxide dismutase (Table 2). However, pretreatment with pregnenolone-16α-carbonitrile, a different microsomal enzyme inducer, greatly potentiated the irreversible binding of 2-hydroxyestradiol, whereas, cobaltous chloride, an inhibitor of microsomal cytochrome P-450 synthesis but not of cytochrome c reductase activity, markedly decreased the binding (Table 2). Heat inactivation of the microsomal enzymes or lack of oxygen almost abolished the binding reaction (Table 2). The binding was inhibited by a carbon monoxide:oxygen atmosphere (9:1) by an antibody against NADPH-cytochrome c reductase (Table 2). Ascorbic acid inhibited the irreversible binding of 6,7-^{3}H-2-hydroxyestradiol to microsomes by 72% while catalase and sodium benzoate had no effect (Table 1). Two other catechols, epinephrine and catechol itself, inhibited the binding of 6,7-^{3}H-2-hydroxyestradiol by 35% and 40%, respectively, Glutathione, a naturally occurring

sulfhydryl containing tripeptide, almost abolished the binding reaction by formation of glutathione adducts of a reactive metabolite of 2-hydroxyestradiol, a reaction previously observed by others.

Significance to Biomedical Research and to the Program of the Institute: Estrogens are receiving considerable attention in view of emerging evidence for their involvement in neoplastic changes in both hormone-sensitive tissues such as breat and vagina, and in tissues that are not hormone-responsive such as the liver and kidney. Our results demonstrate that estrogens and 2-hydroxyestrogens can be converted by hepatic cytochrome P-450 to potent arylating agents. Whether the reaction mechanisms presented here play a role in the toxicities observed in patients receiving extrogens is unclear at this time.

Proposed Course of Project: Covalent binding of estrogens and 2-hydroxyestrogens to tissues in vivo will be determined as well as the effects of this binding on estrogen receptors.

Publications:

Nelson, S.D., Mitchell, J.R., Dybing, E. and Sasame, H.A.: Cytochrome P-450-mediated oxidation of 2-hydroxyestrogens to reactive intermediates. Biochem. Biophys. Res. Commun., in press.

Table 1

Effect of superoxide dismutase on the irreversible binding of estrogen 5 and 2-hydroxyestrogens to rat liver microsomes

Results are means ± S.E. of 4 determinations.

Conditions	Irreversible Binding (moles/mg/min)					
	Estradiol[a]	2-Hydroxy-estradiol	Estrone	2-Hydroxy-estrone	17α-Ethinyl-estradiol	2-Hydroxy-17α-ethinylestradiol
Control (air atmosphere)	0.343 ± .019	0.795 ± .062	0.273 ± .029	1.20 ± .038	0.333 ± .022	1.47 ± .065
- NADPH	0.052 ± .010	0.128 ± .020	0.030 ± .005	0.150 ± .015	0.045 ± .006	0.221 ± .017
+ Superoxide Dismutase (15 ug/ml)	0.294 ± .015[b]	0.578 ± .023[b]	0.228 ± .017[b]	0.780 ± .045[b]	0.281 ± .016[b]	1.19 ± .030[b]
+ Xanthine/Xanthine oxidase (- NADPH)	0.018 ± .003	0.892 ± .055	0.036 ± .023	1.45 ± .089	0.030 ± .005	1.10 ± .098
+ Xanthine/Xanthine oxidase + superoxide dismutase (-NADPH)	0.012 ± .005	0.018 ± .007	0.039 ± .013	0.168 ± .011	0.022 ± .010	0.398 ± .034

[a]Control incubations mixtures (3 ml) contained rat liver microsomal protein (2 mg/ml); phosphate buffer, pH 7.4 (83 mM); estrogen or 2-hydroxyestrogen (0.25 mM); and a NADPH-generating system (NADP, 0.64 mM, glucose-6-phosphate, 15.5 mM; glucose-6-phosphate dehydrogenase, 2 U1/ml; $MgCl_2$, 10 mM). Reactions under air for 10 minutes with shaking at 37° C.

[b]$p < 0.05$ with respect to control as determined by student's t test.

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Table 2

Effects of pretreatments and conditions on the irreversible binding of 6,7-^{3}H 2-hydroxyestradiol (0.25 mM) to rat liver microsomes *in vitro*

Results are means ± S.F. of 4 determinations

Pretreatment and conditions	Irreversible binding (nmoles/mg/min)
A. Complete[a] (air atmosphere)	0.795 ± .062
+ Superoxide dismutase (15 ug/ml)	0.578 ± .023[b]
Phenobarbital	0.779 ± .039
+Superoxide dismutase (15 ug/ml)	0.638 ± .028[b]
Pregnenolone-16α-carbonitrile	1.44 ± .076
+ Superoxide dismutase (15 ug/ml)	1.18 ± .045
Cobaltous chloride	0.386 ± .029
+ Superoxide dismutase (15 ug/ml)	0.285 - .017
B. Microsome (heated 80° x 10 min)	0.032 ± .020
- O_2 (100% N_2 atmosphere)	0.055 ± .010
+ N_2:O_2 (9:1 atmosphere)	0.774 ± .055
+ CO:O_2 (9:1 atmosphere)	0.232 ± .018
+ Preimmune γ- globulin[b]	0.890 ± .010
+ Immune γ- globulin[b] (NADPH cytochrome c reductase antibody)	0.338 ± .062
C. Complete[a] (air atmosphere)	0.795 ± .062
+ Ascorbic acid (0.5 mM)	0.221 ± .026
+Catalase (0.1 mg/ml)	0.804 ± .075
+ Sodium benzoate (5 mM)	0.785 ± .034
+ Epinephrine (0.5 mM)	0.513 ± .025
+ Catechol (0.5 mM)	0.474 ± .012
+ Glutathione (0.5 mM)	0.095 ± .030

[a]Incubation mixtures with a NADPH-generating system as described in Methods and Table 1.
[b]$p < 0.05$ with respect to control as determined by student's t test.
[c]Incubations were as described in Methods. Irreversible binding is expressed as nmoles/mg microsomal protein/min.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00883-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mass Spectral Proof of Gas Chromatographic Assay for 16β-OH-DHEA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	R.B. Franklin	Visiting Fellow	LCP NHLI
	S.D. Nelson	Staff Fellow	LCP NHLI
	J.R. Mitchell	Chief, Section on Clin. Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, MIGMS.

LAB/~~BRANCH~~

Laboratory of Chemical Pharmacology

SECTION

~~Clinical Pharmacology~~

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

The steroid 16β-hydroxydehydroepiandrosterone (16β-OH-DHEA) has been postulated to be important in the pathogenesis of low renin essential hypertension. An assay method using gas chromatography (GC) has been developed to quantiate the excretion and secretion rate of this steroid in human urine samples from normal and hypertensive patients. We have verified the GC method using gas chromatography-mass spectrometry (GC-MS). To verify the accuracy of quantitating 16β-OH-DHEA by the GC method we will synthesize a stable isotope standard of the steroid, add it to extracted urine samples and monitor the parent ions of the steroid and the internal standard as they elute from the GC column into the mass spectrometer.

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PHS-6040
(12-75)

Project Description:

Objectives: The steroid 16β-hydroxydehydroepiandrosterone (16β-OH-DHEA) has been postulated to be important in the pathogenesis of low renin essential hypertension. A gas chromatographic method has been developed to quantitate the excretion and secretion rate of this steroid in urine samples from normal and hypertensive patients. To verify the specificity and accuracy of this assay, we are using a stable-isotope derivative, CG-MS procedure.

Methods Employed: The procedure for isolation and derivitization of the steroid has been outlined in another report ( ). The me-methyloxime-trimethylsilyl derivative (MO-TMS) of 16-oxo-17β-OH-DHEA, 16β-OH-DHEA, and 16α-OH-DHEA were injected onto a glass column (2 mm id x 2 m, 3% SE-30 on 100/120 gas chrom Q) using the same operating conditions described previously (He low 12 ml/min, inj. 270°, column 215°). The column was housed in a Varian Aerograph Model 1400 which was coupled to a V.G. Micromass 16F mass spectrometer. The interface temperature was 270° and source temperature 280°C. The mass spectrometer was operated in the electron impact mode at an accelerating voltage of 4 KV, ionizing current 100 μA and electron energy 70 eV. An integrated ion chromatogram was recorded in a mass range 50-750 a.m.u. and also from 473-481 a.m.u. Normal mass spectra were recorded as each peak eluted into the ion source. Samples prepared from 2 human urine specimens (1 from a control subject and 1 from a low renin hypertensive patient) were also analyzed by GC-MS in a similar manner.

Major Findings: GC-MS of the standards established the retention time of the MO-TMS derivative of the desired 16β-OH-DHEA (46.2 min) relative to other possible isomeric steroids (39.1 min and 50.1 min) and also ensured that good spectra could be obtained as the compounds eluted into the mass spectrometer. GS-MS analysis of the two urine samples showed the presence of 16β-OH-DHEA in both control and hypertensive subjects. Excretion of 16β-OH-DHEA from the control subject appeared to be lower than the excretion from the hypertensive subject.

Significance to Biomedical Research and to the Program of the Institute: A completely specific assay for 16β-OH-DHEA in human urine samples has not previously been developed. To ascertain the role of this steroid in hypertension we have now developed and verified a GC assay using GC-MS.

Proposed Course of Project: To verify the accuracy of quantitating 16β-OH-DHEA we will synthesize the deuterated MO-TMS derivative of 16β-OH-DHEA and we will add a known amount of this derivative as an internal standard to the derivatized steroid mixture isolated from urine samples. This mixture will be subjected to GC-MS and the parent ions of the MO-TMS derivative of 16β-OH-DHEA and its deuterated internal standard will be monitored.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00884-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Covalent Binding of α-methyldopa to Human Red Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI
	E. Dybing	Fogarty International Fellow	LCP NHLI
	S.D. Nelson	Staff Fellow	LCP NHLI

COOPERATING UNITS (if any)
Dr. Dybing was a Fogarty International Fellow supported by the Fogarty International Center. Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

Many reports have described a high incidence of a positive red cell antiglobin reaction (direct Coombs test) in patients taking the antihypertensive drug, α-methyldopa. Based on our previous work showing that cytochrome P-450 generated superoxide anion oxidized α-methyldopa to a reactive arylating agent, we were interested to see if human erythrocytes could activate the drug in a similar manner. Incubation of intact or lysed human red cells with ^{3}H-α-methyl- led to covalent binding of the drug to red cell constituents. Further studies showed that oxyhemoglobin was required for the binding reaction. Because oxyhemoglobin can be visualized as a ferrihemoglobin-superoxide anion complex similar to the monoreduced form postulated for oxygenated cytochrome P-450 in the liver, we believe that superoxide anion from oxyhemoglobin oxidizes the catechol nucleus of α-methyldopa to semiquinone radicals or quinones which covalently bind to the erythrocytes. The denatured macromolecules may then be antigenic and lead to the production of a direct Coombs test and immune hemolysis. The mechanism for this drug allergy is now being studied by attempting to determine the nature of the antigenic component.

PHS-6040

Project Description:

Objectives: We have previously postulated that α-methyldopa is oxidized by cytochrome P-450-generated superoxide anion to a reactive arylating agent which might possibly be a causative factor in chronic active liver disease initiated by therapy with α-methyldopa. Many reports have also described a high incidence of a positive red cell antiglobin reaction (direct Coombs test) in patients taking this antihypertensive drug. In fact, this is probably the most commonly occurring adverse drug reaction yet known. The overall incidence is at least 15% of patients treated with α-methyldopa and about 1% also develop autoimmune hemolytic anemia. No mechanism for this toxic reaction has been established. We were, therefore, interested to see if human erythrocytes could activate α-methyldopa to a potent arylating agent that might react with erythrocyte membranes and macromolecules.

Methods Employed: Venous blood samples from human volunteers were drawn into heparinized tubes and the red cells were isolated by centrifugation. Cells were washed 3 times in buffered isotonic saline. Some samples were resuspended and used intact; other samples were lysed in 20 volumes of water by heating at 37°C for 5 min.

Incubations were carried out at 4°C or at 37°C for varying time intervals. Reactions were started by the addition of ^{3}H-α-methyldopa L-3(3,4-dihydroxyphenyl)-2-methylalanine-3-^{3}H, Amersham-Searle 0.5 mM, sp. act. 2500-3000 dpm/nmole in 1 ml 0.1 M PO_4 buffer pH 7.4 to 1 ml of intact or lysed cells. Reactions were stopped by adding 1 ml 30% trichloroacetic acid (TAC). The protein pellet was washed exhaustively with 5% TCA until no further radioactivity could be extracted. The extracted protein was dissolved in 1.0 ml 1N NaOH and an aliquot counted. Radioactivity was corrected for background and quenching (external standardization) and expressed as nmoles irreversibly bound per mg protein. Modifications to this procedure are described in Table 1.

Major Findings: Incubation of intact or lysed human red cells with ^{3}H-α-methyldopa led to the covalent binding of the drug to red cell constituents with lysed cell binding about six times more than whole cells. The binding was very rapid since no increase was seen with time. Binding to boiled lysates was 5% of values compared to unboiled samples.

In order to evaluate the chemical mechanism underlying the activation of α-methyldopa to an intermediate that binds covalently to red blood cells, we carried out the series of experiments described in Table 1. These binding experiments showed that oxyhemoglobin was required for activation of α-methyldopa both by lysed and intact erythrocytes. Because oxyhemoglobin can be visualized chemically as a ferrihemoglobin-superoxide anion complex similar to the monoreduced form we have postulated for cytochrome P-450 in the liver, we believe that superoxide anion from oxyhemoglobin oxidizes the catechol nucleus of α-methyldopa to semiquinone radicals or quinones which covalently bind to the erythrocyte macromolecules. The denatured macromolecules may then be antigenic and lead to the production of/[a]direct Coombs test

and immune hemolysis.

Significance to Biomedical Research and to the Program of the Institute: We believe that the results reported here make an oxidation product of α-methyldopa a likely candidate for sensitizing red cells in vivo, either by serving as a hapten or by exposing or denaturing antigenic macromolecules in the erythrocytes. If this hypothesis can be shown to be correct, it will be the first documentation of the mechanism underlying a drug-induced allergic reaction.

Proposed Course of Project: We plan to determine the constituents of red cells to which the α-methyldopa is binding. We might then be able to synthesize or purify enough of this constituent to assess its antigenic potential.

Publications:

Mitchell, J.R. and Jollow, D.J.: Role of metabolic activation in chemical carcinogenesis and in drug-induced hepatic injury. In Gerok, W. and Sickinger, R. (Eds.): Drugs and the Liver. Stuttgart, Germany, F.K. Schattauer Verlag, 1975, pp. 395-416.

Mitchell, J.R. and Potter, W.Z.: Drug metabolism in the production of liver injury. Medical Clinics of N. Amer. 59: 877-885, 1975.

Mitchell, J.R., Nelson, S.D., Thorgeirsson, S.S., McMurtry, R.J. and Dybing, E.: Metabolic activation: Biochemical basis for many drug-induced liver injuries. Progress in Liver Disease, Vol. V, in press.

Thorgeirsson, S.S., Sasame, H.A., Mitchell, J.R., Jollow, D.J. and Potter, W.Z.: Biochemical changes after hepatic injury from toxic doses of acetaminophen or furosemide. Pharmacology, in press.

Thorgeirsson, S.S., Mitchell, J.R., Sasame, H.A. and Potter, W.Z.: Biochemical changes after hepatic injury by allyl alcohol and N-hydroxy-2-acetylaminofluorene. Chemico-Biological Interactions, in press.

Mitchell, J.R.: Drugs and the liver. In Tyor, Malcolm P. (Ed.): Viewpoints on Digestive Diseases. Durham, N.C., American Gastroenterological Assoc., Vol. 6, no. 5, No. 1974.

Mitchell, J.R.: Covalent binding: Useful index of the formation of chemically reactive metabolites. Presented at Symposium on Drug-Protein Binding, Univ. of Liverpool, England. Biochem. Sec. Trans. 3: 622-626, 1975.

Mitchell, J.R.: Importance of dose-threshold for drug-induced tissue lesions. Proceedings of Sixth International Congress of Pharmacology, IUPHAR, Helsinki, Finland, 99-108, 1975.

Mitchell, J.R., Hinson, J.A. and Nelson, S.D.: Glutathione and drug-induced tissue lesions. In Arias, I.M. and Jakoby, W. (Eds.): Glutathione: Metabolism and Function. Raven Press, pp. 357-367, 1976.

Dybing, E., Mitchell, J.R., Nelson, S.D. and Gillette, J.R.: Metabolic activation of methyldopa by cytochrome P-450-generated superoxide anion. Proceedings of satellite sympoium on Active Intermediates of the Sixth International Congress of Pharmacology, Turku, Finland, 1975, in press.

Mitchell, J.R., Snodgrass, W.R. and Gillette, J.R.: The role of biotransformation in chemical-induced liver injury. Environmental Health Perspectives, in press.

Mitchell, J.R., Nelson, W.L., Potter, W.Z., Sasame, H.A. and Jollow, D.J.: Metabolic activation of furosemide to a chemically reactive hepatotoxic metabolite. J. Pharmacol. Exper. Therap., in press.

Dybing, E., Nelson, S.D., Mitchell, J.R., Sasame, H.A. and Gillette, J.R.: Oxidation of α-methyldopa and other catechols by cytochrome P-450-generated superoxide anion: Possible mechanism of methyldopa hepatitis. Molec. Pharmacol., in press.

Table 1: Conditions for irreversible binding of ^{3}H-methyldopa to lysed human erythrocytes

Group	Conditions	Irreversible binding nmoles/mg
	^{3}H-methyldopa 0.5 mM control	1.52 ± 0.03
	+ 100% O_2	1.85 ± 0.16
A	+ 100% CO	0.27 ± 0.02
	+ 100% N_2	0.34 ± 0.05
	FerriHgb	0.73 ± 0.06
	+ Ascorbic acid 1 mM	0.09 ± 0.01
B	+ Suproxide dismutase 15 micro g/ml	1.57 ± 0.11
	+ Catalase 0.2 mg/ml	1.46 ± 0.03
C	+ KCN 0.1 M	5.12 ± 0.17
	+ NaN_3 0.1 M	0.21 ± 0.01
	+ Ethylenediamine 20 mM	1.44 ± 0.06
D	+ Glutathione 1 mM	0.35 ± 0.05
	+ Dopa 0.5 mM	0.19 ± 0.05
	+ Dopamine 0.5 mM	0.67 ± 0.07
E	+ Epinephrine 0.5 mM	1.20 ± 0.11
	+ Norepinephrine 0.5 mM	1.19 ± 0.02
	+ Catechol 0.5 mM	0.44 ± 0.03
	+ 3-O-Methyldopa 0.5 mM	1.69 ± 0.19
	^{3}H-Dopa	2.27 ± 0.12
	^{3}H-Dopamine	1.65 ± 0.14
F	^{3}H-Epinephrine	2.26 ± 0.17
	^{3}H-Norepinephrine	1.70 ± 0.14
	^{14}C-3-O-Methyldopa	0.49 ± 0.02

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00885-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Evaluation of potentially toxic N-hydroxyamide metabolism of lidocaine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	S.D. Nelson	Staff Fellow	LCP NHLI
Other:	W.F. Trager	Faculty Member	Univ. of Wash.
	W.L. Nelson	Faculty Member	Univ. of Wash.
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)
Drs. W.F. Trager and W.L. Nelson are faculty members of the Dept. of Pharmaceutical Chemistry, Univ. of Washington, Seattle, Washington.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

SUMMARY OF WORK (200 words or less - underline keywords)

Lidocaine is a widely used anesthetic and antiarrhythmic agent. One of its major metabolites in man is the mono- N-deethylated product MEGX. Preliminary evidence was recently presented that N-hydroxylidocaine and N-hydroxyMEGX are also major metabolites in man. Because of the carcinogenic propensity of other N-hydroxyamides, we have synthesized the potential N-hydroxyamide metabolites and tested for their formation in normal subjects using chemical ionization mass spectrometry and stable isotope labeling. We have also determined the mutagenic potential of the metabolites using the Ames' test and the carcinogenic potential in mice. Results of these experiments indicate that the N-hydroxyamides are not major metabolites of lidocaine in man and that they are neither mutagenic nor carcinogenic based on the testing procedures described. We are now attempting to determine whether these metabolites are formed in patients receiving the drug.

PHS-6040
(12-75)

Project No. Z01 HL 00885-01 LCP

Project Description:

Objectives: Lidocaine (1) is a widely used anesthetic and antiarrhythmic agent. One of its major metabolites in man is the mono-N-deethylated product MEGX (2). Preliminary evidence was recently presented that N-hydroxy lidocaine (3) and N-hydroxy MEGX (4) are also major metabolites in man (Mather and Thomas, Life Sci. Part 1, 11, 915-919 (1972)). Because of the carcinogenic propersity of other N-hydroxyamides, several scientists have voiced concern over the widespread use of lidocaine and its possible toxicity. Therefore, we have synthesized the potential metabolites, tested for their formation in normal subjects using chemical ionization mass spectrometry (CIMS) and stable isotope labeling, determined their mutagenic potential using the Ames test, and determined their carcinogenic potential in mice. A final objective of the project is to establish whether these metabolites are formed in patients receiving iv infusions of lidocaine in coronary care units.

Methods Employed: The two N-hydroxyamide metabolites 3 and 4 were prepared by reacting chloroacetyl chloride with 2,6-dimethylphenylhydroxylamine in a slurry of aqueous sodium bicarbonate in ether. The ω-chloro-N-hydroxyamide thus formed reacted with the appropriate amine to give the desired N-hydroxyamide. Deuterated standards were prepared in a similar manner. Urine was collected for 24 hours from 3 volunteers receiving 250 mg doses of lidocaine and known amounts of deuterated standards were added. Aliquots of the urine samples were alkalinized and extracted. CIMS analysis of the extracts was then performed.

Carcinogenicity testing was performed through the National Cancer Inst. testing program. The Ames test for mutagenicity was performed both with and without liver S-9 fraction using tester strain TA-1538 which is known to be sensitive to other N-hydroxyamides. 2-Acetylaminofluorene (2-AAF) was used as a positive control.

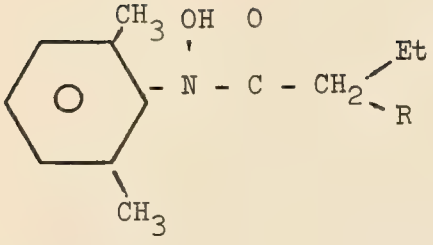
Major Findings: Based on the CIMS-stable isotope analysis, no N-hydroxyamide metabolites of lidocaine or MEGX could be found in the urine of three subjects receiving lidocaine. Carcinogenicity tests showed that these N-hydroxyamides were noncarcinogenic in mice and the mutagenesis assays showed no revertant colonies above background levels, whereas the positive control, 2-acetylaminofluorene, produced 5500 colonies.

Significance to Biomedical Research and to the Program of the Institute: Based on these results, there appears to be no reason to voice concern about potential mutagenicity or carcinogenicity from this widely used and life-saving cardiac drug.

Proposed Course of the Project: To ensure that patients receiving up to 24 hour iv infusions of lidocaine do not form these metabolites, we will be analyzing plasma and urine samples from such patients who are being treated in a coronary care unit.

Publicatons: None

STRUCTURES



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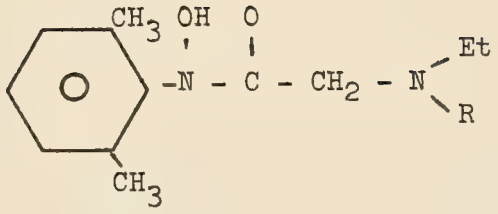
2, R = H



3, R = Et

4, R = H

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00886-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Identification of cysteine and GSH conjugates of reactive metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	S.D. Nelson	Staff Fellow	LCP NHLI
Other:	J.R. Mitchell	Chief, Sec. on Clinical Pharm.	LCP NHLI
	J.A. Hinson	Staff Fellow	LCP NHLI
	M.R. Boyd	Research Assoc.	LCP NHLI
	A. Buckpitt	Guest Worker	LCP NHLI

COOPERATING UNITS (if any)

Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS
Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.
Dr. Buckpitt holds an NIH Research Fellowship.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Many drugs and other chemicals that are ingested by man are metabolized to reactive intermediates that bind to tissue macromolecules and ultimately produce tissue lesions. Because these metabolites are very reactive, we are developing indirect methods for elucidating their structures. These methods involve trapping the intermediates formed in microsomal incubations with cysteine or glutathione, chromatographic isolation of the glutathione and cysteine conjugates, and structure elucidation of the purified conjugates using mass spectrometry and nmr spectroscopy. The techniques have been successfully applied to various hydrazines, α-methyldopa and 2-hydroxy-estradiol. Other compounds which form reactive intermediates such as the furans and furan drug, furosemide, and analgesic drugs, acetaminophen and phenacetin, are presently being investigated.

PHS-6040
(12-75)

Project Description:

Objectives: Many xenobiotics, including drugs, can be metabolized to reactive intermediates that bind to tissue macromolecules and ultimately produce tissue lesions. Since these metabolites are extremely reactive, elucidation of their structures is possible only by indirect means. Methods are being developed for trapping these intermediates with naturally occurring sulfhydryl reagents, for isolation of the conjugates formed, and for elucidation of their structures using mass spectrometry and nmr spectroscopy.

Methods Employed: Many xenobiotics that cause tissue lesions in man and other animals are converted by enzymes in the endoplasmic reticulum in the target organ to reactive metabolites that covalently bind to tissue macromolecules *in vivo* and *in vitro*. *In vitro* reactions are carried out by incubating radiolabeled substrates, microsomes, varying concentrations of an NADPH-generating system and trapping reagents (cysteine or glutathione). In some cases stable isotope-labeled substrates are used to follow particular reaction pathways. Covalent binding to microsomes is determined on the precipitated protein by methods already described by this laboratory, and the supernatant is lyophilized. The desired conjugates are isolated by a combination of Sephadex, ion-exchange and thin-layer chromatographic procedures. Methods using chemical ionization mass spectrometry (VG MicroMass 16F instrument) are being developed to determine the structures of these conjugates. Some conjugates require derivatization to enhance their volatility and the advantages and disadvantages of various derivatives are being explored using S-methylglutathione as a reference compound. If enough conjugate is available ($\sim$500 µg) Fourier transform PMR spectra (Varian XL-100 instrument) is utilized to elucidate further the structure of the conjugates.

Major Findings: 1) Hydrazines. a) Acetylhydrazine. This hepatotoxic metabolite of isoniazid, a widely used tuberculostatic drug, covalently binds to rat liver microsomes. The binding is decreased by the addition of either glutathione or cysteine with the concomitant formation of S-acetylglutathione and N-acetylcysteine, respectively, as determined by chemical ionization mass spectrometry of the isolated conjugates. A twin-ion study was carried out with a 1:1 mixture of non- and tri-deuterated acetylhydrazine. Chemical ionization mass spectra of the isolated N-acetylcysteine showed the same ratio of hydrogen to deuterium as the substrate mixture. Since no deuterium was lost during the activation process, the reactive metabolite cannot be ketene.

b) Isopropylhydrazine. This hepatotoxic metabolite of iproniazid, an antidepressant removed from clinical use because of its hepatotoxicity, also covalently binds to liver protein, a reaction which is similarly decreased by the addition of sulfhydryl compounds. Samples of both the cysteine and glutathione conjugates have been isolated from this reaction but not enough to obtain good mass spectra. Additional experiments are now in progress using mixtures of isopropylhydrazine and its C-2 deuterated analog.

c) Monomethylhydrazine. This known lung carcinogen is a component of rocket fuels. Experiments are now in progress using non- and trideutero-methylhydrazine to determine more about its possible activation pathway.

2) α-Methyldopa. This widely used antihypertensive agent causes both hepatic and hemoprotein disorders in patients receiving the drug. Trapping experiments with glutathione after activation of the drug by rat liver microsomes or human red blood cells (oxygenated hemoglobin) have shown that a glutathione conjugate is formed with chromatographic and spectral proeprties almost identical to a dopa-glutathione conjugate synthesized by others. Fourier transform PMR indicates that the conjugate is 6-S-glutathionyl-α-methyldopa.

3) Furans. Many furans are lung toxins including 4-ipomeanol, a toxin in moldy sweet potatoes; 3-methylfuran, an air pollutant; 2-methylfuran and furan, products of cigarette smoke; and nitrofurantoin, an antibacterial drug. Furosemide, a widely used furan-containing diuretic drug, can cause kidney and liver lesions. Trapping experiments are now underway with many of these furans. The parent compounds yield readily interpretable EI and CI mass spectra which should prove helpful in elucidation of metabolites and conjugates formed in trapping reactions.

4) 2-Hydroxyestrogens. Estrogens have been implicated implicated in neoplastic development in many tissues. 2-Hydroxyestrogens are metabolites of estrogens which are further oxidized by microsomal enzymes to reactive metabolites that covalently bind to tissue macromolecules. Cysteine and glutathione both block this binding with the formation of conjugates that can be isolated by Sephadex chromatography. Mass spectra of these conjugates indicate the position of conjugation (C-1- and 4-thioethers) as well as the nature of the conjugate thus formed.

5) Analgesics - p-Hydroxyacetanilide (acetaminophen, PHAA) and p-ethoxy-acetanilide (phenacetin, pEAA). PHAA and PEAA in high doses cause liver and kidney lesions in man and animals. Covalent binding of both compounds to tissue macromolecules is decreased by sulfhydryl reagents. Conjugates can be isolated from reactions of both compounds in incubations containing microsomes, NADPH-generating system and glutathione. Enough conjugate for mass spectral analysis is forthcoming.

Significance to Biomedical Research and to the Program of the Institute: These results provide information about the nature of toxic reactive metabolites of useful drugs and other xenobiotics. Elucidation of the structures and physical properties of these toxic intermediates is fundamental to our attempts to decrease toxicity by appropriate changes in therapeutic regimens.

Proposed Course of Project: Further studies are to be carried out as described in Major Findings.

Publications:

Nelson, S.D., Hinson, J.A. and Mitchell, J.R.: The use of chemical ionization mass spectrometry and the twin-ion technique to better define a mechanism in acetylhydrazine toxicity. Biochem. Biophys. Res. Commun. 69(4), 900-907, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00887-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
A Mass Spectral Assay for Propranolol and its Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	S.D. Nelson	Staff Fellow	LCP NHLI
	W.L. Nelson	Faculty Member	Univ. of Wash.
Other:	D.R. Rollins	Research Assoc.	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)
Dr. Nelson is a Staff Fellow and Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, MIGMS. Dr. W. Nelson is a faculty member of the University of Washington, Seattle, Wash.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

SUMMARY OF WORK (200 words or less - underline keywords)

Propranolol is a widely used β-blocking agent which has been found useful in the treatment of hypertension. We are developing a sensitive and specific assay for propranolol to assess the specificity of existing procedures and extend the assay to include several metabolites of the drug. To accomplish this we are synthesizing deuterated analogues of propranolol and its metabolites for use as stable isotope internal standards for gas chromatography-mass spectrometry. Based on our initial results with plasma extracts of propranolol and propranolol d_5, selected ion monitoring will be necessary for a sensitive and reproducable assay.

PHS-6040
(12-75)

Project Description:

Objectives: Propranolol is a widely used β-blocking agent which has been found to be useful in the treatment of hypertension. To study the fate of this drug in hypertensive patients we are developing a sensitive assay to assess the specificity of existing procedures (i.e. fluorometric) and to extend the assay to include metabolites which may have pharmacological activity in their own right, either efficacious or toxicological.

Methods Employed: The following deuterated internal standards have been synthesized: propranolol-d_5 (I), and 2-hydroxy-3-(1-naphthoxy)-propranolol-d_5 (II). Mass spectral analysis was performed by direct insertion probe on a VG Micromass 16F instrument using the following conditions: 1) Electron ionization (EI) accelerating voltage 4kV, electron energy 70 eV, emission current 100 μA, source temperature 200°C. 2) Chemical ionization (CI) accelerating voltage 4 kV, electron energy 100 eV, emission current 200 μA, source temperature 200°C. isobutane reactant gass, source pressure 0.3 Torr.

Major Findings: Mass spectral analysis of the deuterated propranolol and propranolol metabolite verifies their structure and shows them to be > 98% pentadeuterated. The EI spectra show substantial (>15%) molecular ions plus several peaks due to fragment ions. The CI spectra show that 80% of the total ion current is carried by the quasimolecular (M+1) ions. This would suggest that CI might be the most sensitive technique for quantifying propranolol and its metabolites.

Small amounts (10-50 ng) of propranolol and propranolol-d_5 were added to fresh human blood samples and plasma separated by centrifugation. The plasma was extracted with ether and the ether extract carefully evaporated under nitrogen. The residue from evaporation was then dissolved in methanolic HCl. EI mass spectrometry was not sensitive enough to detect the propranolol or propranolol-d_5. CI mass spectrometry of the samples showed propranolol and its deuterated internal standard to be present but only sufficient sample for one scan due to the extreme volatility of the sample.

Significance to Biomedical Research and to the Program of the Institute: The metabolism and pharmacokinetics of propranolol has not been studied in hypertensive patients. Because the doses employed in the treatment of hypertension are often greater than those previously prescribed for propranolol, determinations of metabolites and their quantities should be made in individuals receiving such therapy.

Proposed Course of Project: Chemical ionization mass spectrometry appears to provide a sensitive technique for monitoring propranolol and its metabolites. However, for low leveles of the metabolites in plasma extracts, gas-chromatography coupled with either electron or chemical ionization mass spectrometry and selected ion monitoring appears essential. These techniques will be attempted in the near future. The use of deuterated internal standares ensures that propranolol and its metabolites will be specifically quantified. Additional deuterated standards are now being prepared.

Project No. z01 HL 00887-01 LCP

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00888-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Deuterium isotope effects in O-deethylation reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	S.D. Nelson	Staff Fellow	LCP NHLI
	H.A. Sasame	Chemist	LCP NHLI
Other	W.A. Garland	Senior Scientist	Hoffmann-La Roche, Nutley, N.J.

COOPERATING UNITS (if any)
Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS. Dr. Garland is in the Biochemistry and Drug Metabolism Section of Hoffmann-La Roche, Nutley, N.J.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Results accumulated over the past several years have shown that there are significant deuterium isotope effects in oxidative dealkylation reactions when deuterium is substituted for hydrogen at the reacting center or an adjacent center. Other studies have shown that substitution with stable isotopes can decrease the carcinogenic potential of various carcinogens such as nitrosamines. We have therefore embarked on a program to determine the effect of such substitution on the toxicity of various drugs which might yield toxic metabolites by dealkylation. Such a study is under way with the analgesic drug phenacetin. Preliminary studies in vitro using chemical ionization mass spectrometry as an assay technique have revealed a significant deuterium isotope effect when deuterium was substituted for hydrogen at the reacting center (k_H/k_D 2). Additional studies are in progress to determine the possible effects in vivo of this deuterium substitution as well as the effects of deuterium substitution on the β-methyl group.

PHS-6040
(12-75)

Project Description:

Objectives: Previous work has shown that there are significant isotope effects on N- and O-demethylation reactions and N-deethylation reactions when deuterium is substituted for hydrogen at the reacting center or an adjacent center. Other studies have shown that such substitution can decrease the carcinogenic potential of certain known carcinogens such as nitrosamines. We have therefore embarked on a program to see if such substitution can decrease the toxicity of certain drugs which might be partially activated by O-dealkylation mechanisms. Such a study is under way with the analgesic drug, phenacetin (PEAA).

Methods Employed: p-Ethoxy-α,α'-d_2-acetanilide (PEAA-d_2) and p-ethoxy-β,β',β"-d_3 - acetanilide (PEAA-d_3) were synthesized by alkylation of the sodium salt of p-hydroxyacetanilide (PHAA) with ethyl-α,α'-d_2-bromide and ethyl-β,β',β"-d_3 bromide, respectively. The products were recrystallized from methanol:water mixtures. Microsomes were isolated from rabbit liver and the kinetic parameters for the O-dealkylation reaction were determined for PEAA and its di- and trideuterated analogs using standard incubation procedures. After each reaction was stopped, a known amount of N-acetyl-d_3-p-aminophenol was added to each incubation flask as an internal standard. The products of the reaction were extracted into ether and the ether extract methylated with diazomethane. The products were analyzed by gas chromatography-chemical ionization mass spectrometry and quantified by selected ion monitoring of the parent ions of pHAA and pHAA-d_3.

Major Findings: PEAA-d_2 showed an isotope effect (k_H/k_D) at V_{max} of approximately 2, indicating that its rate of dealkylation was considerably slower under the conditions of the assay, whereas PEAA-d_3 showed no effect. The K_m for dealkylation was unchanged by deuterium substitution.

Significance to Biomedical Research and to the Program of the Institute: These preliminary experiments show that if dealkylation of PEAA is necessary for eliciting the toxic response to this drug, then the toxicity might be decreased by α-deuterium substitution.

Proposed Course of Project: First an animal model must be developed for the renal toxicity observed in patients receiving PEAA. Then the effect of α-deuterium substitution on the toxicity will be studied in vivo. Mechanistic information might then be derived from further studies in vitro. The effect of β-deuterium substitution will be studied on another possible toxic metabolite of phenacetin, p-(2-hydroxyethoxy)-acetanilide.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00889-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Synthesis of potentially toxic metabolites of salicylates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	S.D. Nelson	Staff Fellow	LCP	NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP	NHLI

COOPERATING UNITS (if any)
Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology, Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

SUMMARY OF WORK (200 words or less - underline keywords)

Salicylates have been implicated in several toxic reactions in man including hepatic and renal necrosis and analgesic nephropathy. We have undertaken the synthesis of potentially toxic metabolites of salicylic acid in order to help elucidate the mechanism of these toxicities. Radiolabeled ^{14}C-gentisic acid has been prepared and initial studies have shown that it covalently binds to microsomal protein. Additional syntheses and experiments in vivo and in vitro with gentisic acid and other metabolites of salicylic acid are now being carried out.

PHS-6040
(12-75)

Project Description:

Objectives: Salicylates produce dose-related renal and hepatic necrosis in man. Elucidation of the mechanism leading to this toxic reaction requires the synthesis of radiolabeled metabolites of possible importance in the pathogenesis of the tissue injury.

Methods Employed: Published synthetic procedures were employed using commercially available reagents and radiolabeled compounds. All radioactive syntheses were preceded by "cold" synthesis of enough material for melting point, NMR, and mass spectral analysis. Radiochemical purity of the prepared derivatives was determined by recrystallization to constant specific activity followed by thin-layer chromatography. Melting points were determined on a Thomas-Hoover Uni-melt instrument, NMR on a Varian A-60, and mass spectra on a VG MicroMass 16F instrument operated in both electron and chemical ionization modes.

Major Findings: ^{14}C-Carboxyl-labeled gentisic acid was synthesized by oxidation of ^{14}C-carboxyl-labeled salicylic acid with potassium persulfate in basic solution. A preliminary reaction *in vitro* with rat liver microsomes showed it to be covalently bound to the microsomal protein. The binding required oxygen and NADPH.

Significance to Biomedical Research and to the Program of the Institute: Salicylates are widely used drugs in this and other countries. We have synthesized gentisic acid, a potentially toxic metabolite of salicylates in the hope of eventually elucidating the mechanism for salicylate-induced kidney and liver injury. Rational approaches to therapy are impossible without this fundamental knowledge of biochemical mechanisms.

Proposed Course of Project: Additional syntheses of other metabolites are in progress. Studies in animals will be carried out with sodium salicylate and metabolites to learn more about the toxic pathways involved.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00890-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemical and Morphological Correlates in Drug-Induced Lung Injury

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.R. Boyd	Research Associate	LCP NHLI
OTHER:	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.2	0.4

SUMMARY OF WORK (200 words or less - underline keywords)

Although the covalent binding of chemically reactive metabolites with pulmonary tissues has been clearly implicated in the process of chemical lung injury, the precise mechanisms of chemical/tissue interactions in the pathogenesis of the lesions has not been elucidated. In this project we are approaching these questions using a combination of biochemical measures coupled with morphologic techniques, including high-resolution light microscopy, electron microscopy, and autoradiography.

PHS-6040
(12-75)

Project Description:

Objectives: Pulmonary injury produced by ingested agents, including therapeutic agents in humans, is being recognized with increasing frequency. Recent studies from our laboratory have shown that certain chemical agents, such as 4-ipomeanol (IPO) (See other reports), may produce lung injury due to their metabolism to alkylating agents in the lung. Although the covalent binding of chemically reactive metabolites with pulmonary tissues has been clearly implicated in the process of chemical lung injury, the precise mechanisms of chemical/tissue interactions in the pathogenesis of the lesions have not been elucidated. These investigations were therefore addressed to the latter question.

In these studies we have initially used 4-ipomeanol as a model agent which produces a selective pulmonary toxicity and which requires metabolic activation in the expression of its toxicity.

Methods Employed: 1) Experimental animals. Studies have employed male, HLA-SD rats (Hilltop), HLA-SW mice (Hilltop), C57BL/6J mice (Jackson), and Golden Syrian hamsters (NIH).

2) Radiolabeled IPO. ^{14}C-IPO (1-2 mCi/mmole) was obtained as described in another report. ^{3}H-IPO (generally labeled; 12-56 mCi/mmole) was obtained by catalytic tritiation of IPO with T_2O, using a palladium catalyst in trifluroacetic acid. The purified product was then subjected to repeated exchange in methanol to remove labile tritium. (^{14}C-IPO and ^{3}H-IPO gave similar patterns of *in vivo* covalent binding, both quantitatively and qualitatively, as measured by our standard assay).

3) Preparation of tissues. Lungs have been prepared for conventional light microscopy (LM) using standard methods; sections were stained with hematoxylin and eosin. Samples for high resolution light microscopy (HRLM) and electron microscopy (EM) were obtained from lungs perfused and fixed *in situ* with 3% glutaraldehyde buffered with sodium cacodylate; the samples were embedded in methacrylate resin prior to sectioning. HRLM samples were stained with toluidine blue; EM specimens will be stained with uranyl acetate.

4) Morphologic localization of IPO. Autoradiography (ARG) is being used to study the cellular and subcellular localization of covalently bound radiolabeled IPO. ARG is being done with both ^{3}H-IPO and ^{14}C-IPO with the LM and HRLM sections. Only ^{3}H-IPO will be used in the EM-ARG studies. Presumably, only covalently bound radioactivity will be detected in the ARG studies, since unbound toxin should be removed during the dehydration and preparation of the tissues for microscopy. Appropriate experiments will be done to confirm this.

Major Findings: 1) Dose-dependencies of biochemical and morphological parameters. Rats were dosed (ip) with either ^{14}C-IPO (1 mCi/mmole) or ^{3}H-IPO (12 mCi/mmole) in a range of doses between 5 and 40 mg/kg. Animals were sacrificed after 6 hr and lungs were worked up for LM, HRLM, EM, and LM-ARG

in the usual way. By LM and HRLM no striking changes were seen at this early (6 hr) time interval. Previous biochemical studies showed that covalent binding of IPO in the lung is maximal by 2 hr after dosing. Representative EM examinations ot tissues from each dose level are in progress. A preliminary study was done to determine optimal dark times for ARG. HRLM samples from the high dose animals were dipped in Kodak NT2B emulsion and developed for 2, 6, 8, 10 and 12 weeks. From these studies, optimal dark times were determined to be 10 weeks for the ^{3}H-IPO and 15 weeks for the ^{14}C-IPO. All specimens have now been dipped and are presently being developed.

The ARG specimens obtained in the latter preliminary study indicate that the cells with the heaviest labeling are the bronchiolar lining cells and the alveolar type II cells. If confirmed in subsequent studies, this finding may be of considerable significance. It is reasonable to speculate that these cells are selectively alkylated by certain chemicals due to their ability to convert them to reactive metabolites. It is interesting that these two cell types represent the cellular origin of 90% of all pulmonary cancers.

2) Time-course studies. Rats and mice were dosed (ip) with 20 and 30 mg/kg, respectively, of ^{3}H-IPO or ^{14}C-IPO. They were sacrificed after 1/2, 1, 2, 4, 8, and 16 hr and the lungs prepared for HRLM and EM in the usual way. Examination of the HRLM sections reveals that earliest histological changes visible at the light microscopic level, begin to appear 4-8 hr after dosing. There is slight thickening of the alveolar walls, and some swelling of the type II pneumocytes. At later intervals, the lamellar bodies of the type II cells increase in number and become swollen and quite prominent. Early nuclear changes are apparent in bronchiolar cells by 4 hr; the bronchiolar cells eventually become necrotic. By 8 hr there is significant perivascular, peribronchiolar, interstitial, and intraalveolar edema. By 16 hr there is complete loss of the normal integrity of the lung; there is widespread multicellular necrosis, hemorrhage and consolidation.

EM studies of representative specimens from each time interval are in progress. We hope thereby to detect critical initial morphologic alterations occurring at the early time intervals.

Specimens for ARG at each time interval have been dipped and are currently being prpcessed. We anticipate that the ARG studies will provide the essential data for correlating the key biochemical events (i.e., metabolic activation and covalent binding) with the subsequent morphological sequellae.

3) Subcellular localization of covalently bound IPO. Once the target cells for IPO are conclusively identified it will be of considerable interest to determine the essential chemical/cellular interactions that result in individual cell death. We are approaching this question using the special technique of electron microscopic autoradiography (EM-ARG). High specific activity ^{3}H-IPO is necessary for these studies. The following species were dosed (ip) with ^{3}H-IPO (56 mCi/mmole): Rat (7 and 20 mg/kg) mice (Swiss and C57BL/6J; 10 and 30 mg/kg). The animals were sacrificed after 2 hr and the lungs were processed in the usual way for LM, HRLM, and EM. Preliminary

LM-ARG studies are in progress to verify the cellular localization of IPO. Representative specimens will be selected for EM-ARG to determine the subcellular localization of IPO in the target cells.

4) In order to validate the ARG studies we have dosed (ip) rats and mice pretreated with piperonyl butoxide (1000 mg/kg, s.c.) and $COCl_2$ (20 mg/kg bidx2d, s.c.) with ^{3}H-IPO and ^{14}C-IPO. Preparation of the tissues for HRLM-ARG is in progress. Biochemical studies showed that IPO toxicity and covalent binding are decreased by these MFO inhibitors, although the tissue levels of free IPO are actually increased. Thus ARG studies of tissues of these animals should reflect only the decreased levels of covalently bound IPO and not the increased levels of unbound IPO.

Significance to Biomedical Research and the Program of the Institute: We feel these studies will contribute significantly to the understanding of critical chemical/tissue interactions involved in the pathogenesis of pulmonary disease produced by drugs and other chemicals in the environment.

Proposed Course of Project: Most of this experimental work with IPO is now complete. The data are presently being processed and interpreted. We hope to extend these studies to include other pulmonary toxins, particularly α-naphthylthiourea, nitrofurantoin and 3-methylfuran.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00891-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of the Metabolism and Toxicity of 1-Nitronaphthalene

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.R. Boyd	Research Associate	LCP NHLI
	L.R. Pohl	Guest Worker	LCP NHLI
OTHER:	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP NHLI

COOPERATING UNITS (if any)
Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Pohl is a GuestWorker supported by an NIH Research Fellowship.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

SUMMARY OF WORK (200 words or less - underline keywords)

We are presently investigating the biochemical basis for the production of hepatic and pulmonary necrosis by 1-nitronaphthalene (NN) in rats. Elucidation of the role for reductive activation in the pathogenesis of tissue lesions produced by compounds such as NN may contribute to our understanding of adverse reactions produced by therapeutic agents containing the nitro moiety.

PHS-6040
(12-75)

Project Description:

Objectives: We have studied the possibility that certain drugs and other environmental chemicals are converted to reactive metabolites that produce serious pulmonary renal and hepatic toxicities. Most of our previous studies have dealt with the metabolic activation of toxins by oxidative pathways.

Several toxic and carcinogenic metabolites are hydroxylamine derivatives, which can be formed *in vivo* either by oxidation of amines or by reduction of nitro compounds.

A recent report [Tox. Appl. Pharmacol. 33, 132 (1975)] indicated that 1-nitronaphthalene (NN) causes pulmonary edema and hepatic necrosis in rats. Furthermore, 1-naphthylamine has been isolated as a urinary metabolite of NN in rats, indicating 1-nitronaphthalene is reduced *in vivo*. These observations stimulated our present interest in studying the possible role of reductive activation in the pathogenesis of hepatic and pulmonary lesions by 1-nitronaphthalene.

Methods Employed: Standard procedures are being used.

Major Findings: 1) Synthesis. We have synthesized 8-^{14}C-NN by nitrating 8-^{14}C-naphthalene with fuming nitric acid and have purified it by preparative TLC or high-pressure liquid chromatography. Unlabeled 1-nitronaphthalene is commercially available (Eastman).

2) Toxicity of 1-nitronaphthalene. We have examined the toxicity of 1-nitronaphthalene in male, HLA-SD, specific pathogen free rats. The compound was given ip in corn oil in doses of 50-800 mg/kg. The LD_{50} (24 hrs) was approximately 300 mg/kg. With the 300 mg/kg dose, pronounced pathological changes were seen in the livers and lungs. Centrilobular hepatic necrosis was apparent; the lungs showed widespread multicellular necrosis, hemorrhage, and edema. At doses of 80 mg/kg significant pulmonary damage was seen (especially bronchiolar necrosis), but hepatic necrosis was not present. Pretreatment of rats with antibiotics, to reduce the gut flora, did not alter the toxicity. Experiments are underway to determine the effects of various inducers and inhibitors of both oxidative and reductive metabolic pathways on the possible metabolic activation, and the toxicity of 1-nitronaphthalene.

Significance to Biomedical Research and the Program of the Institute: Elucidation of the role for reductive activation in the pathogenesis of tissue lesions produced by compounds such as 1-nitronaphthalene may contribute to our understanding of adverse reactions produced by nitro-containing therapeutic agents such as nitrofurantoin.

Proposed Course of Project: Both *in vitro* and *in vivo* studies will be undertaken to determine if toxic metabolites are produced from 1-nitronaphthalene and to evaluate the relative contribution of various metabolic pathways in their production under a variety of experimental conditions.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00892-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of the Metabolism and Toxicity of Aminohydantoin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	M.R. Boyd	Research Associate	LCP NHLI
	J.S. Dutcher	Guest Worker	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Dutcher is on leave from the Dept. of Pharmacology, Northwestern University, where he is supported by an NIH Postdoctoral Fellowship.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

In earlier studies a variety of substituted hydrazines were found to produce liver injury due to their further metabolic conversion, in vivo, to acylating or alkylating agents. There is precedent to suggest that aminohydantoin (AH) could be formed in vivo as a degradation product from either nitrofurantoin or Dantrium. Since AH can be viewed as a substituted hydrazine, we are investigating it as a possible causative factor in hepatic damage produced in patients by these drugs.

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PHS-6040
(12-75)

Project Description:

Objectives: Recent studies from this laboratory have demonstrated that acyl and alkyl hydrazines may be metabolites of such drugs as isoniazid and iproniazid and may be responsible for the hepatotoxicity produced by these drugs in patients. A variety of substituted hydrazines have been found to produce diffuse hepatocellular necrosis in experimental animals due to their further metabolic conversion, in vivo, to acylating or alkylating agents.

Dantrium (5-p-nitrophenyl-2-furfurylidine aminohydantoin) (DT) is a drug recently marketed for the treatment of severe muscular spasticity. Unfortunately the drug produces an alarmingly high incidence of severe and sometimes fatal hepatic injury. Similarly, there are several recent reports that an another structurally related drug, nitrofurantoin (5-nitro-2-furfurylidine aminohydantoin) (NF), may also cause severe liver injury, in addition to its well-known toxic effects on the lung. We feel that key structural features commom to both DT and NF is the aminohydantoin moiety. There is considerable precedent to suggest that the -C = N- linkage of both DT and NF could be cleaved under acidic conditions found in the gut; in both cases, acid cleavage would yield free aminohydantoin (AH). Further precedent is available to suggest that AH would be absorbed and readily acetylated in vivo to form N-acetylaminohydantoin (AAH). Both AH and AAH may be viewed as hydrazine derivatives possibly subject to metabolic activation, analogous to the mode of activation of other toxic hydrazines.

We have therefore undertaken the present studies to evaluate the hypothesis that AH and/or AAH are possible metabolites which may be responsible for production of toxic lesions by DT and NF. Our initial approach has been to prepare AH and AAH synthetically and to examine their metabolism and toxicity in experimental animals.

Major Findings: Neither AH or AAH are available commercially and it was therefore necessary to develop an appropriate synthesis; it was also necessary that the synthesis should be amenable to the preparation of both (acetyl-^{14}C)-AAH and (hydantoin-^{14}C)-AH to be used in the metabolic studies. Benzylidine aminohydantoin (BA) was prepared by the reaction of benzaldehyde semicarbazone with ethylbromoacetate and sodium ethoxide. AH hydrochloride was obtained after refluxing BA with dilute HCl. Free AH was extracted from an aqueous solution of AH-HCl after addition of potassium carbonate. AAH was obtained from AH by reaction with acetic anhydride. Analyses by mass spectroscopy and nuclear magnetic resonance confirmed the identity of the products. (Acetyl-^{14}C)-AAH and (hydantoin-^{14}C)-AH are presently being prepared using ^{14}C-ethyl-bromoacetate and ^{14}C-acetic anhydride, respectively.

A variety of experimental animal species, both before and after phenobarbital pretreatment, have been administered a range of doses of both AH and AAH. The tissues are presently being processed for histological examination.

Significance to Biomedical Research and to the Program of the Institute: The elucidation of the mechanism(s) of production of toxic lesions by DT and NF in experimental animals should provide the basis for a more rational approach to the prevention and/or treatment of such toxic reactions in man.

Proposed Course of Project: If the present studies support the hypothesis that AH or AAH can be metabolically activated to a toxic species, further investigations will be initiated to determine if AH or AAH are formed in vivo from DT and/or NF and could thereby account for production of tissue lesions by these drugs. Also, since many hydrazine derivatives are known carcinogens, studies of the possible carcinogenicity of AAH may also be indicated.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00893-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of Mechanisms of Development of Tolerance to Chemical Injury of the Lung

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. R. Boyd	Research Associate	LCP NHLI
OTHER:	H.A. Sasame	Chemist	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

The development of pulmonary tolerance and cross-tolerance to lung-toxic agents is a well-recognized but poorly understood phenomenon. In this project we are using 4-ipomeanol (IPO) as a model pulmonary toxin to study biochemical and morphological changes accompanying tolerance development. It appears that principles established with IPO may have general applicability to other pulmonary toxicants.

PHS-6040
(12-75)

Project Description:

Objectives: In studies initiated to evaluate chronic changes produced by the pulmonary toxin, 4-ipomeanol, it became evident that pretreatment of experimental animals with small ip or iv doses of the toxin produces a state of remarkable tolerance to subsequent challenge with normally lethal doses of the compound given by any route. A search of the literature revealed that tolerance development to a number of lung-toxic agents, including both inhaled materials as well as compounds given by other routes, is a well-recognized phenomenon. However, although rather extensively studied, the mechanisms of tolerance induction by these agents remain obscure.

The latter findings stimulated our present interests in mechanisms of development of tolerance to chemical lung injury. We are employing 4-ipomeanol (IPO) as a model pulmonary toxin in most of the present studies since it is one of the most specific chemical lung toxins known, and we have recently acquired much insight into its molecular mode of action (see other progress reports).

Methods Employed: We are using standard procedures, or those described in earlier publications, or in other progress reports. All studies have been done with HLA-SD rats (Hilltop), unless otherwise indicated. "Tolerant" rats have been obtained by pretreating normals for 7 days with 8 mg/kg of IPO unless otherwise indicated.

Major Findings: 1) Covalent binding of IPO, in vivo. Since earlier studies demonstrated that the toxicity of IPO is due to metabolism of the compound to an alkylating agent in the target tissue, we examined the covalent binding of IPO in control and tolerant rats. Covalent binding of radioactivity after administration of ^{14}C-IPO (doses of 10-80 mg/kg) was markedly reduced in lungs and livers of tolerant rats, compared to controls.

2) Effect of IPO pretreatment on hepatic and pulmonary mixed-function oxidases. Earlier studies showed that IPO pretreatment (12 mg/kg ip x 7 days) caused a reduction in hepatic and pulmonary microsomal benzphetamine demethylase and aniline hydroxylase activities, and in the covalent binding of IPO in vitro. This suggested that the decreased covalent binding and toxicity of IPO in tolerant rats might be due to decreased formation of a chemically reactive metabolite. However, we recently found that pretreatment of rats with a lower dose (5 mg/kg) of IPO for 7 days does not alter hepatic or pulmonary benzphetamine demethylase, aniline hydroxylase, or cytochrome P-450 levels, although it does induce a significant degree of tolerance to IPO toxicity. This suggests that a decreased rate of formation of a toxic metabolite in tolerant rats cannot totally account for the tolerance. Other possibilities therefore must also be considered, such as alterations in the ratios of toxication and detoxication pathways available to IPO (and/or its metabolites), or alterations in the inherent sensitivity of the lung chemically reactive metabolites.

3) Glutathione (GSH) levels in tolerant rats. No significant differences in hepatic and pulmonary levels of GSH were found in control and tolerant rats, suggesting tolerance is not due to increased GSH availability. The possibility of an alteration in GSH-transferase activities cannot be eliminated though.

4) Morphologic studies. We are examining the morphologic features of lungs of tolerant rats compared to controls. On gross examination, the lungs of tolerant rats appear somewhat larger than normal; they weigh approximately 10-20% more than normal, and their total protein content is increased to a similar extent. Microscopic examination of hematoxylin and eosin stained, paraffin-embedded sections of lungs of tolerant rats reveals no edema, congestion, hemorrhage, or other features associated with the acute toxic response to IPO. High resolution light microscopy of toluidine blue-stained, methacrylate-embedded sections reveals a striking difference in the lungs of tolerant and control rats. In the tolerant lungs, two important features are noted: 1) there is a marked proliferation of type II pneumocytes; they are not only increased in number, but also in size and in their content of lamellar bodies (containing phospholipid surfactant); 2) the bronchiolar lining cells have undergone squamous metaplasia. Other studies are presently in progress to further characterize the tolerant lungs at an ultrastructural level, using electron microscopy. In addition, animals have been dosed with high specific activity of ^{14}C-IPO or ^{3}H-IPO at both acutely toxic (mg/kg) and tolerance-inducing (7 mg/kg) levels in order to determine if the cellular and subcellular localization of covalently-bound IPO is similar at the two dose levels and if the morphologic changes associated with the two doses are actually qualitatively different, or if they only differ quantitatively. Autoradiographic (ARG) techniques, at both the light- and electron-microscopic levels are being utilized to localize the bound toxin.

In other studies we are similarly comparing the morphologic changes and the cellular and subcellular localization of covalently bound IPO in lungs of control and tolerant rats given normally lethal challenge doses (25 mg/kg) of ^{14}C-IPO and ^{3}H-IPO. Preliminary examinations of tissues from these animals indicates that the low-dose pretreatment with IPO almost completely prevents the acute toxic changes (observed by light microscopy) produced by the challenge dose. The electron-microscopic examinations and ARG studies of these tissues are not yet complete.

5) Cross-tolerance phenomena. We have found that rats made tolerant to IPO by low-dose IPO pretreatment are also markedly tolerant to other systemically administered pulmonary toxins, such as α-naphthylthiourea (ANTU), as well as inhaled gases that produce lung injury, such as 100% oxygen and ozone. This illustrates that tolerance produced by IPO is nonspecific and may therefore represent a general adaptative mechanism of the lung.

Significance to Biomedical Research and to the Program of the Institute: The phenomenon of tolerance development to the pulmonary toxin, 4-ipomeanol, may be a reflection of a fundamental defense mechanism of the lung, which serves to protect it from chemical injury both from the external (atmospheric)

environment as well as from the internal (systemic) environment. Furthermore, metabolic activation, occurring in the lung, may be an important mechanism underlying the pathogenesis of lung diseases resulting from exposure to a variety of xenobiotics. Therefore, mechanisms of tolerance induction elucidated with the model lung toxin, IPO, may be generally applicable to the well-known, but poorly understood, tolerance and and cross-tolerance phenomena produced by a variety of lung-toxic chemicals and gases.

Proposed Course of Project: In future studies we will continue to focus our attention upon the correlation of toxicological, biochemical, and morphologic changes in pulmonary tolerance development. Emphasis will be placed particularly on the evaluation of changes in toxication and detoxication pathways for toxins such as IPO. We will attempt to further clarify the role of altered cellular populations in the lung in the induction of tolerance. Finally, we hope to determine if principles established with model toxins like IPO can be extended to other pulmonary toxicants in the environment.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00894-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Role of Glutathione as a Modulator of Drug-Induced Pulmonary Toxicities

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.R. Boyd	Research Associate	LCP NHLI
OTHER:	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)
Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

It is now apparent that highly reactive electrophilic metabolites may be involved in the causation of certain chemically-induced pulmonary diseases. The present studies suggest that the endogenous nucleophilic tripeptide, glutathione (GSH), may combine with these toxic metabolites to form nontoxic, excretable conjugates. GSH may thereby play an important role in protecting the lung against potential injury by xenobiotics.

PHS-6040
(12-75)

Project Description:

Objectives: Previous investigations in this laboratory have demonstrated a fundamental role of endogenous glutathione (GSH) as a "protective" factor in certain drug-induced hepatic and renal toxicities. Highly reactive, electrophilic intermediates, formed during metabolism of some xenobiotics, can react with the nucleophilic GSH to form nontoxic, excretable conjugates. In the absence of GSH, chemically reactive metabolites may combine covalently with nucleophilic tissue components to produce pathological alterations.

It is now apparent that chemically reactive metabolites may also be involved in the production of pulmonary disease. Therefore, we are investigating the possibility that GSH may play an important role in regulating susceptibility to certain lung toxicities produced by drugs or other environmental chemicals.

Methods Employed: Standard procedures are being used. HLA-SD rats have been used in all studies reported herein. Present studies have employed 4-ipomeanol (IPO) nitrofurantoin (NF), and α-naphthylthiourea (ANTU) (Boyd and Neal, Drug Metab. Disp., in press), all of which produce pulmonary toxicities in experimental animals.

Major Findings: 1) In vitro. With lung or liver microsomes, both glutathione (GSH) and cysteine (CYS) inhibit covalent binding of IPO, NF, and ANTU without inhibiting their metabolism. This indicates that the added nucleophilic reagents can trap the reactive metabolites formed from metabolism of these compounds in vitro. Preliminary studies indicate that the reactive metabolite formed from IPO in vitro can react both directly with GSH and also by an enzyme ("soluble fraction")-catalyzed process.

2) GSH depletion, in vivo. Toxic doses of IPO (10-40 mg/kg) deplete lung GSH by 15-45%, without significantly affecting GSH levels in liver or kidney. GSH depletion and covalent binding are proportionate to the dose of IPO given; no threshold effect for GSH depletion or covalent binding, is apparent. Administration of piperonyl butoxide, one hour before IPO, inhibits metabolism, covalent binding, toxicity, and GSH depletion by IPO. 3-Methylcholanthrene pretreatment changes the target organ (from lung to liver) for covalent binding, toxicity, and GSH depletion by IPO. These findings indicate that a toxic metabolite, formed by metabolism of IPO in vivo may deplete tissue GSH in vivo.

ANTU (10 mg/kg, i.p.) or NF (100 mg/kg, s.c.) did not significantly alter GSH levels in lung.

3) Effect of diethylmaleate and cysteine on in vivo covalent binding and toxicity. Diethylmaleate (DEM) was used to deplete tissues of endogenous GSH. DEM caused a striking increase in both the covalent binding (lung and liver) and the toxicities of IPO and ANTU.

Cysteine pretreatment, which blocks several other drug-induced toxicities,

presumably by increasing GSH synthesis or by acting directly as an alterate nucleophile, inhibits both the toxicity and the covalent binding of IPO. These findings suggest that endogenous GSH may act as an alternate nucleophile to react with toxic metabolites of IPO and ANTU.

Significance to Biomedical Research and to the Program of the Institute:

The present studies indicate that endogenous GSH may play an important role in protecting the lung against certain types of chemical injury.

Proposed Course of Project: We are continuing to study the possible role of GSH in drug-induced lung disease, using the model pulmonary toxin, 4-ipomeanol. We also plan to extend these studies to include inhaled toxicants such as 3-methylfuran.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00895-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of the Pathogenesis of Nitrofurantoin-Induced Lung Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. R. Boyd	Research Associate	LCP NHLI
OTHER:	J. R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)
Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI-NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

We are able to reproducibly elicit lethal pulmonary lesions with nitrofurantoin (NF) in rats, in a dose-dependent fashion. This indicates that NF-induced lung disease in man may be the result of a direct pulmonary toxicity, and not an immune response as is widely believed. Other studies indicate that NF can be metabolized to highly reactive chemical species; we are, therefore, investigating the possibility that metabolic activation is involved in the production of lung lesions by NF.

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PHS-6040
(12-75)

Project Description:

Objectives: The 5-nitrofuran derivative, nitrofurantoin (NF), is widely used in the treatment of urinary tract infections. It is also used, on a more limited scale, for long-term prophylaxis against certain urinary-tract pathogens. Unfortunately, the drug sometimes produces serious, and potentially fatal, lung disease in patients. Both acute and chronic pulmonary reactions have been described. The acute form is characterized by a respiratory distress syndrome; chest x-rays usually show pulmonary infiltrates and effusions. The chronic reaction is more insidious in onset and clinical and radiologic manifestations are consistent with a diagnosis of pulmonary fibrosis. Lung biopsies have confirmed the fibrotic pulmonary changes.

Although these abnormal responses to NF have been widely assumed to represent hypersensitivity phenomena, immune mechanisms have not been clearly implicated. Furthermore, neither the clinical spectra of the diseases, nor the organ specificities, are accountable simply as allergic reactions.

We have undertaken the present investigations to evaluate the possibility that nitrofurantoin produces a direct, dose-related pulmonary toxicity. We are also investigating the possibility that the toxicity is due to metabolism of the parent drug to a chemically reactive metabolite. We have chosen this approach since other nitrofurans are known to be converted to cytotoxic and carcinogenic species by nitroreduction; furthermore, many furan derivatives, not containing the nitro moiety, are converted by oxidative pathways to toxic metabolites that may produce lesions in livers, kidneys, and lungs of experimental animals.

Methods Employed: 1) Experimental animals. Male, specific pathogen free, HLS-SD rats, obtained from Hilltop, Inc., are being used in these studies. We have found these animals to be particularly suitable for studies of drug-induced pulmonary lesions; they are completely free of the chronic respiratory infections frequently seen in many rat colonies.

2) ^{14}C-Nitrofurantoin. Radiolabeled NF is essential for many of the studies described herein. The labeled compound unfortunately is not available commercially. We have therefore explored several approaches for obtaining the compound. A synthetic method for (hydantoin-^{14}C)-labeled NF has been developed using ^{14}C-ethylbromoacetate (Amersham-Searle) as the source of the label. Development of a synthesis for (formyl-^{14}C)-NF is in progress. A small amount of the latter derivative has been kindly supplied by Norwich Pharmaceutical Co.

3) In vitro studies. Microsomal and soluble-fraction enzyme preparations have been obtained from lung and liver using standard methods. Soluble-fraction preparations have been further treated by chromatography on Sephadex G-25 to remove endogenous cofactors and glutathione (GSH). Aerobic and anaerobic incubations, and assays for covalently bound radioactivity, have been done according to conventional procedures. Total reductive metabolism of NF in incubations has been measured by the loss of absorbance

at 400 nm.

4) In vivo studies. Assays for radioactivity covalently bound to tissues in vivo have been done conventionally. Pulmonary toxicity has been assessed histologically and also by lung wet weight/dry weight ratios and lung weight/body weight ratios. Lungs have been prepared for conventional light microscopy using standard methods. Samples for high-resolution light microscopy and electron microscopy were obtained from lungs perfused and fixed in situ with 3% glutaraldehyde buffered with sodium cacodylate; the samples were embedded in methacrylate resin prior to sectioning.

Major Findings: 1) In vitro studies. Both lung and liver microsomes contain enzyme systems which catalyze covalent binding of ^{14}C-NF to microsomal protein. Both the reduction of the nitro group and the covalent binding occur maximally under anaerobic conditions and require NADPH as a cofactor. The binding is not blocked by carbon monoxide, and is markedly stimulated by flavins. The enzyme is, therefore, similar to NADPH cytochrome c reductase. These data indicate that metabolic activation of NF in lung and liver microsomes in vitro occurs primarily by nitroreduction and not by oxidation of the furan ring.

Soluble enzymes of both lung and liver also convert NF to a chemically reactive metabolite. As before, both nitroreduction and covalent binding are greatest under anaerobic conditions. Maximal activities are observed with NADH or hypoxanthine as cofactors, suggesting that one activating enzyme is xanthine oxidase. Some activity was also observed with N-methylnicotinamide or NADPH as cofactors, also suggesting the possible participation of aldehyde oxidase and/or a diaphorase.

The nitroreduction of NF by the soluble or microsomal fractions is not inhibited by the addition of GSH. In contrast, GSH markedly inhibits covalent binding of NF. This suggests that the reactive metabolite is a highly electrophilic species which can react with nucleophilic cellular constituents or, alternatively, with mobile nucleophiles such as GSH to form soluble conjugates.

2) In vivo studies. ^{14}C-NF binds covalently (100-700 pmoles/mg protein) in vivo to various tissues of the rat, especially in kidney, liver and lung after i.v. doses of 1.5-90 mg/kg. Since earlier in vitro studies demonstrated that NF is nonreactive without prior metabolism, the present studies indicate that some NF is also converted in vivo to an alkylating agent. The relative contribution of various metabolic pathways (and also metabolism by gut flora) in the production of chemically reactive NF metabolites, or the possible significance of such metabolites in the pathogenesis of NF-lung, are as yet unknown.

Most significantly, we are able to elicit reproducibly lethal pulmonary lesions in rats with NF, given subcutaneously in doses of 100-400 mg/kg. Several hours after dosing, the rats show progressive dyspnea, but no cyanosis.

In rats given lethal doses, cyanosis does appear shortly before death and frothy fluid is often seen dripping from the nostrils at the time of death. At post-mortem examinations the lungs grossly appear hyperinflated, hemorrhagic, and edematous. Lung wet weight/dry weight and lung weight/body weight ratios are elevated. Microscopically, there is vascular hemorrhage and widespread intraalveolar, perivascular, and peribronchiolar edema. Emphysematous changes are also present, especially in the parenchyma nearest the pleural surfaces. Necrosis of a variety of cell types can also be identified. No significant pathological changes are seen in other organs using light microscopy. Pretreatment of the animals with antibiotics to reduce the gut flora did not reduce the toxic response to NF.

Significance to Biomedical Research and the Program of the Institute: The observation of a reproducible and dose-related pulmonary toxicity with NF in experimental animals suggests that NF-induced lung disease in man may be the result of a direct pulmonary toxicity; it may, therefore, be dependent upon a variety of pharmacokinetic factors, rather than an immune response. The availability of the experimental model for NF-induced disease described here should not only aid the elucidation of the mechanism of the reaction, but also may provide the basis for development of a more rational approach to the prevention and/or treatment of the disease in humans.

Since NF can be metabolized to highly reactive chemical species, it is reasonable to speculate that metabolic activation is involved in the pathogenesis of acute NF-induced lung lesions. However, an additional implication should be emphasized. The finding that NF alkylates tissues *in vivo* suggests that a metabolite such as an N-hydroxylaminofuran may be formed from NF *in vivo*. The latter type of metabolite is presumed to be responsible for the carcinogenicity of many 5-nitrofurans. Although NF is a known mutagen, it is commonly assumed to be noncarcinogenic, and therefore, is presumably somewhat unique among the nitrofurans. However, the latter conclusion appears to be based on extremely limited published data obtained from very small groups of animals, and of only a single species. In view of the present findings, and the widespread use of NF in humans, we feel that NF should perhaps be re-evaluated as a potential carcinogen.

Proposed Course of Project: Studies are underway to further characterize lung lesions, both acute and chronic, produced by NF in rats. These studies will employ both high-resolution light microscopy as well as electron microscopy, and hopefully will provide further insight into the pathogenesis. Further metabolic studies will also be undertaken to probe the possible role of metabolic activation in NF-induced lung disease.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00896-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Metabolic Activation in the Pathogenesis of Lung Injury by 4-Ipomeanol

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. R. Boyd	Research Associate	LCP NHLI
OTHER:	H. A. Sasame	Chemist	LCP NHLI
	S. D. Nelson	Staff Fellow	LCP NHLI
	J. R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)
Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.
Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

This project was undertaken to elucidate the biochemical basis for the acute lung toxicity of 4-ipomeanol (IPO), a naturally occurring furan derivative. It is now apparent IPO produces pulmonary necrosis due to its metabolism to an alkylating agent in the target tissue. In continuing studies we are investigating the toxication and detoxication pathways available to IPO and the chemical nature of its toxic electrophilic metabolite(s).

PHS-6040
(12-75)

Project Description:

Objectives: 4-Ipomeanol (IPO) (1-[3-furyl]-4-hydroxypentanone) is a poisonous metabolite produced in mold-damaged sweet potatoes. It produces a pronounced and specific pulmonary toxicity in a variety of laboratory and domestic animals. The lung lesions are characterized principally by widespread edema, congestion, and multicellular necrosis.

The ingestion of mold-damaged sweet potatoes has been implicated for many years in natural outbreaks of poisoning in cattle. Affected animals suffer severe and often fatal respiratory distress. Pathological findings are usually restricted to the lungs. IPO is apparently the etiological agent in this peculiar disease.

IPO, and other toxic components, frequently have been detected in minimally blemished sweet potatoes offered for sale at food markets, although the public health significance of these observations is not known. It is of interest that certain populations, such as several tribes in the highlands of New Guinea, whose principal dietary component is the common sweet potato, have an extraordinarily high incidence of both acute and chronic pulmonary disease. Furthermore, our recent finding that IPO specifically alkylates the lung *in vivo* [Boyd *et al.*, Tox. Appl. Pharm. 32, 147 (1975)] has stimulated considerable interest in IPO as a potential dietary carcinogen. A federal contract has been issued for carcinogenicity testing of the compound.

The present project was undertaken to elucidate the biochemical basis for the acute lung toxicity of this interesting furan derivative. It is the continuation of a project that Dr. Boyd initiated while at another institution.

Previous studies showed that, after administration of ^{14}C-IPO to rats, radioactivity is covalently bound to several tissues. The amount bound in lung is 2-3 fold higher than in any other tissue. The covalent binding and the toxicity of IPO are due to its conversion to a chemically reactive metabolite that alkylates the target tissue. *In vitro* studies indicate that a reactive metabolite of IPO is formed by cytochrome P-450-dependent microsomal mixed-function oxidases (MFO) in both lung and liver microsomes. Pretreatment of rats with MFO inhibitors *in vivo* cause parallel reductions in covalent binding and toxicity, suggesting that MFO-mediated metabolism of IPO is responsible for its covalent binding and toxicity *in vivo*. Pretreatment of rats with 3-methylcholanthrene (MC) increases the rate of formation of the toxic metabolite in the liver; in MC-treated rats IPO produces liver necrosis, but minimal lung damage. This suggests the toxic metabolite is extremely reactive and does not escape the organ in which it is formed. A logical conclusion, therefore, is that the pulmonary toxicity of IPO results from pulmonary metabolism of the compound.

The following experiments have been undertaken to supplement and to clarify further the interpretation of the previous studies.

Methods Employed: Standard procedures, or those which have been described in earlier publications or in other progress reports, were used. Male, HLA-SD rats (Hilltop) have been used in all studies unless otherwise indicated.

Major Findings: 1) Improved method for preparation of lung microsomes. We have developed a new procedure for routine preparation of lung microsomes containing cytochrome P-450 of 2-3 times greater specific activity than has been previously reported. The key modifications are the use of 15% glycerol in the homogenization medium, and performance of the initial homogenization under an N_2 atmosphere with N_2-purged media.

2) Effect of $CoCl_2$ pretreatment on lung cytochrome P-450. Previous studies indicated that pretreatment of rats with cobaltous chloride causes a reduction in liver cytochrome P-450, but a paradoxical increase in P-450 of the ileum. Its effect on P-450 content of other tissues has not been documented. Since we found earlier that $CoCl_2$ treatment inhibits the covalent binding and toxicity of IPO *in vivo*, we examined its effects on lung cytochrome P-450. Pretreatment of rats with 30 mg/kg, s.c. of $CoCl_2$ twice daily for two days, causes approximately a 40% decrease in lung P-450. The treatment produces a similar decrease in the magnitude of covalent binding of IPO *in vitro* by lung microsomes.

3) Development of assay for total metabolism of IPO in vitro. Total metabolism of IPO can be measured reliably by monitoring the disappearance of ^{14}C-IPO (specific activity ~500 dpm/nmole). Mixtures containing the appropriate amount of substrate and cofactors and 4 mg of microsomal protein in 2 ml final volume are incubated for various times and the reactions terminated by addition of 2 ml of methanol containing 100 µmoles of unlabeled IPO. A 100 µl aliquot of the supernatant is streaked on a silica plate (5 x 20 cm Analtech SG-F, 250 µ), which has been previously deactivated with methanol. The plate is developed in 20% MeOH/Benzene. The IPO band is located with short-wave u.v. and scraped into a scintillation vial containing 2 ml of methanol and shaken. A 10 ml aliquot of Bray's solution is added and the sample counted (LSC). Gas chromatography and radiochromatography indicate that the IPO band obtained and counted using this procedure contains only IPO and no radioactive metabolites or impurities. Therefore, the disappearance of IPO can be determined by comparing the counts obtained in the test samples with those obtained similarly from incubations containing heat denatured microsomes or with essential cofactors deleted.

4) Comparison of total metabolism (TM) and covalent binding (CB) of IPO. A time course study of TM and CB of IPO was done with liver microsomes. Both the rate of TM and CB are linear only for 4-5 min. By 30 min no additional TM or CB occur. In liver microsomes TM is about 4-5 fold greater than total CB. This is especially interesting since preliminary experiments indicate that TM and CB are approximately equal in lung microsomes. This may help explain the fact that V_{max} values for CB of IPO in lung and liver microsomes are similar (and K_m in lung about 10-fold K_m in liver), although the P-450 levels are much greater in liver. The different TM/CB ratios in lung and

and liver could, in part, be a reflection of differences in activities of potential detoxication pathways in the two tissues (such as epoxide hydrase, in which the lung is known to be relatively deficient).

A method is also being developed for measurement of levels of unmetabolized IPO in whole-tissue homogenates. The method will be used in further experiments to measure, in addition to covalent binding, the total metabolism and the tissue clearance of IPO in animals pretreated with MFO inhibitors or inducers. We anticipate that these experiments will add further validity to the concept that IPO toxicity is due to metabolic activation of the toxin within the target tissue.

5) Nature of reactive metabolite(s) of IPO. An exploratory experiment was done to examine the ability of various nucleophilic reagents to inhibit covalent binding of IPO in vitro. Several agents are effective inhibitors; the order of effectiveness is glutathione > cysteine > phenylmercaptan > mercaptoethanol > cyclohexylmeraptan > propylmercaptan > ethylmercaptan > t-butylmercaptan. From these studies it appeared that glutathione (GSH) or cysteine (CYS) would be most useful as nucleophilic reagents with which to trap an electrophilic metabolite of IPO. CYS was selected for use as the trapping agent in the present study since a much simpler cysteine/IPO conjugate would result than if GSH were used; this would greatly facilitate structural analyses of the conjugate by nmr and mass spectrometry.

Other experiments indicated that CYS does not inhibit microsomal metabolism of IPO. This supports the idea that the inhibition of covalent binding of IPO by CYS is due to formation of a water-soluble conjugate of CYS with a reactive IPO metabolite. Presumably, therefore, structural analysis of the conjugate may provide insight into the nature of the toxic electrophilic metabolite of IPO.

Approximately 800 mg of lung microsomes were obtained from the lungs of 360 rats and incubated in a final volume of 200 ml with ^{14}C-IPO and CYS (both 10^{-3}M), in the presence of an NADPH generating system.

The reaction was stopped by addition of an equal volume of methanol. Preliminary examination (Sephadex chromatography) of a small quantity of the solution indicated a conjugate was indeed formed. Further work is now underway to isolate a sufficient amount of the conjugate for structural studies by both nmr and mass spectrometry. Other metabolites will also be sought. These studies will also be done with liver microsomes from normal, phenobarbital pretreated, and 3-methylcholanthrene-pretreated rats. For the proper interpretation of in vivo experiments utilizing the last pretreatments, it is important to determine if the same electrophilic metabolite is formed under all these conditions, and/or if ratios of toxication/detoxication pathways are significantly altered.

Studies of metabolite patterns will also be carried out in vivo, with and without pretreatments with MFO inhibitors and inducers, in order to determine the nature of the toxic metabolite(s) formed from IPO, and the

nature of the detoxication pathways available to IPO [or metabolite(s)] in vivo.

Significance to Biomedical Research and the Program of the Institute: Chemicals which reproducibly produce an acute, specific pulmonary toxicity by routes of administration other than inhalation are rare and their mechanisms of action are poorly understood. IPO, therefore, may provide a unique tool with which to probe pathogenic mechanisms of chemically-induced lung disease. An understanding of the mode of action of this model agent may provide new insight into both normal and abnormal pulmonary physiology as well as into mechanisms of, and factors regulating susceptibility to, pulmonary disease produced in humans by drugs or other chemicals in the environment.

The present studies may also aid in the evaluation of any potential public health significance of IPO as a possible food contaminant.

Proposed Course of Project: This project is nearing completion; several manuscripts are in preparation.

Publications:

Boyd, M. R.: Role of metabolic activation in the pathogenesis of chemically-induced pulmonary disease; mechanism of action of the lung-toxic furan, 4-ipomeanol. Environmental Health Perspectives, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00897-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthesis of 4-ipomeanol-[^{14}C] of high specific activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. R. Boyd	Research Associate	LCP NHLI
OTHER:	J. R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

A method has been devised for the preparation of the pulmonary toxin, 4-ipomeanol-[^{14}C] (IPO) with a specific activity of 2 mCi/mmole. The compound is necessary in biochemical studies requiring the measurement of very low concentrations of IPO or its metabolites. It is also required for use in autoradiographic studies of the tissue localization of IPO.

PHS-6040
(12-75)

Project Description:

Objectives: For our studies of the mechanism of action of the pulmonary toxin, 4-ipomeanol (IPO), it became necessary to develop a new method for preparation of ^{14}C-IPO of high specific activity. The material is essential for certain biochemical studies of the metabolic activation of the compound both *in vitro* and *in vivo*, which involve very low concentrations of the toxin. High specific activity ^{14}C-IPO is also essential for our ongoing studies of the target cell(s) and morphologic features of the pathogenesis of IPO-induced lung disease. Autoradiographic methods, with ^{14}C-IPO, are being utilized to determine target structures alkylated by IPO.

Methods Employed: Standard methods of semimicro- and micro- scale organic synthesis have been adapted to our needs.

Major Findings: We have successfully prepared ^{14}C-IPO with a specific activity of 2 mCi/mmole, using an adaptation of a previously published procedure [Boyd *et al.*, Nature 236, 158 (1972)]. The method has been optimized for maximum yield of label in a minimum amount of product. The product obtained represents about 100-fold greater specific activity than that produced with previously published methods [Boyd *et al.*, Toxicol. App. Pharmacol. 32, 147 (1975)]. ^{14}C-Propylene oxide (American Radiochemical Corp.) was used as the source of the radiolabel. Sodium methoxide (0.95 mmoles) was prepared by dissolving the appropriate amount of Na metal in a total volume of 0.8 ml of methanol. Ethyl 3-furoylacetate 184 mg (1 mmole); prepared as described in above publications was added and the mixture stirred at room temperature for 10 min. Next, 54 mg (1 mmole) of ^{14}C-propylene oxide (2 mCi total ^{14}C) was added and the reaction vessel was tightly sealed and stirred for 60 hr at 25°. Then, 0.1 ml of 10 N HCl was added and the mixture stirred for 18 hr at 58°. The volume was decreased to 0.2 ml with a stream of N_2. A solution of 100 mg $NaHCO_3$ in 0.2 ml H_2O was added, and the mixture extracted with ether. After drying with $MgSO_4$ the ether was removed and the residue applied to a 20 x 20 cm silica TLC plate (1000 μ). The plate was eluted with 15% MeOH/benzene; the IPO band was removed and extracted with ether. The final step of purification was done similarly, using a 1000 μ silica plate eluted with 50% ethylacetate/hexane. The product was both chemically and radiochemically pure as determined by previously described analytical procedures. The final yield was usually 40-60% of the theoretical amount.

Significance to Biomedical Research and the Program of the Institute: The synthesis described here provides a ready source of high specific activity ^{14}C-IPO which is essential for the further study of the molecular pathology of lung disease produced by this unique and interesting pulmonary toxin.

Proposed Course of Project: The project has been completed; a manuscript is in preparation.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00898-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Genetic Factors in Target Organ Toxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. R. Boyd	Research Associate	LCP NHLI
	J. S. Dutcher	Guest Worker	LCP NHLI
OTHER:	J. R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Dutcher is on leave from the Dept. of Pharmacology, Northwestern Univ., where he is supported by an NIH Postdoctoral Fellowship.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

In this project we are exploring the possibility that species and strain differences can be utilized to help define the biochemical bases for the selective destruction of various tissues by certain chemical agents. The present studies, using 4-ipomeanol as a model toxin, clearly illustrate that genetic and constitutional factors may play a fundamental role in the expression and regulation of target organ toxicities by chemical agents that can be activated by metabolism in a variety of different tissues.

PHS-6040
(12-75)

Project Description:

Objectives: We are exploring the possibility that species and strain differences can be utilized to help define the biochemical bases for the selective destruction of various tissues by certain chemical agents. We are using 4-ipomeanol (IPO) as a model agent, since earlier studies showed it to be a highly selective pulmonary toxin, due to its selective alkylation of the lung by a chemically reactive metabolite formed in the target tissue. It is of interest to determine if different tissue sensitivities or specificities for IPO occur in other species or strains. If significant differences are present they may provide a new experimental avenue for studying metabolic and dispositional factors which may regulate target organ specificities for environmental toxicants in general.

A secondary objective of the present studies is to identify species or strains of animals appropriate for carcinogenicity testing with IPO. Considerable interest has recently developed in 4-ipomeanol as a potential dietary carcinogen. Federal contracts have been issued both for studies of its possible carcinogenicity and for its analysis in food samples. IPO occurs naturally in mold-damaged sweet potatoes.

Methods Employed: Standard procedures are being used.

Major Findings: 1) Toxicity of IPO. In the HLA-SD rat, guinea pig, and rabbit, IPO produced only lung lesions at all doses tested. LD50 values (i.p.) were (mg/kg) 24 $\pm$ 4, 15 $\pm$ 5 and 30 $\pm$ 10, respectively.

In all mouse strains tested, IPO produces both pulmonary and renal necrosis with doses near the LD50 value. With much lower doses it is possible to produce significant pulmonary lesions without affecting the kidney (as assessed by light microscopy). LD50 values (ip) in the different mouse strains tested are as follows: (mg/kg) NIH-GP, 43 $\pm$ 3; BALB/cJ, 60 $\pm$ 5; A/J, 20 $\pm$ 3; C57BL/6J, 25 $\pm$ 5; DBA/2J, 25 $\pm$ 3.

The hamster is strikingly less sensitive to IPO than any other species tested. The LD50 dose (ip) is 140 $\pm$ 10 mg/kg, which is 2-9 times the lethal dose in any other species. Interestingly, doses near the LD50 value produce both lung and liver necrosis. The hamster is the only species in which liver necrosis has been observed with IPO (in noninduced animals). At doses less than 80 mg/kg, however, IPO produces only pulmonary necrosis (by light microscopy).

2) Species and train differences in covalent binding of IPO in vivo. In confirmation of previous studies, radioactivity from toxic ip doses of ^{14}C-IPO became covalently bound selectively to the lungs of the HLA-SD rat (levels of covalent binding: lung > liver > kidney). Covalent binding studies have not yet been done in the guinea pig and the rabbit.

In all mouse strains tested, highest levels of covalent binding are seen in the kidney (levels of covalent binding: kidney > lung > liver). This

is interesting since IPO produces significant renal toxicity in the mouse. Another important point, however, is that low doses of IPO may only damage the lung; this occurs even though the overall tissue concentration of radioactivity, as measured in our crude assay for covalent binding, is higher in kidney than in lung. This is probably a reflection of the much greater homogeneity of cellular population of lung as compared to the kidney. It is probable that the key toxic interactions in the lung affect only certain cell populations. Therefore, the covalently bound toxin in lung may be highly localized and measures of total tissue levels of covalently bound radioactivity may not accurately reflect the relative degree of critical tissue damage when comparing the lung to tissues with much more homogeneous cellular populations, such as kidney and liver.

A comparison of gross tissue levels of covalently bound toxin in the different mouse strains does provide a rough estimate of their relative sensitivities to IPO. For example, the A/J, C57BL/6J and DBA/6J strains are much more sensitive to IPO toxicity (as estimated by LD50 values) than are the NIH-GP and BALB/CJ strains; similarly, covalent binding of IPO to kidneys, livers, and lungs of the more sensitive strains are much higher than with comparable doses in the less sensitive strains.

In the hamster, much lower levels of covalent binding of IPO were seen in all tissues relative to tissues of other species with comparable doses. This agrees well with the relative insensitivity of the hamster to IPO toxicity. The relative levels of covalent binding of IPO to different hamster tissues are in the order liver > lung > kidney. This also is consistent with the finding that IPO produces hepatic lesions in hamsters with doses near the LD50 value. Liver lesions are not seen at lower doses, with which significant pulmonary damage is produced, even though the overall level of binding in liver is greater than in lung. The implications of the latter findings were discussed earlier (see discussion for mouse).

3) Effect of 3-methylcholanthrene (MC) on target organs for IPO in genetically inducible (B6) and non-inducible (D2) inbred mice. MC pretreatment resulted in increase in covalent binding of IPO in the livers of B6 mice, and a reduction in binding to lungs and kidneys. The LD50 value was increased from 25 $\pm$ 5 mg/kg in the controls to 125 $\pm$ 10 mg/kg in the MC-pretreated group. In contrast to the findings with controls, centrilobular hepatic necrosis was seen in the MC-pretreated group given IPO at doses near the LD50 value. In the non-inducible D2 mice, MC had no effect on the toxicity or covalent binding of IPO.

These findings are consistent with our previous conclusion that IPO toxicity results from metabolism of the compound to an alkylating agent within the target tissue. They further illustrate the importance of genetic factors in target organ toxicity.

Significance to Biomedical Research and the Program of the Institute:

These studies clearly illustrate that genetic and constitution factors may

play a fundamental role in the expression and regulation of target organ toxicities by chemical agents that can be activated by metabolism in a variety of different tissues.

Proposed Course of Project: We are proceeding with further studies, both in vivo and in vitro, to determine if metabolic and dispositional factors can account for the species and strain differences in the relative sensitivities and target organ specificities of IPO.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00899-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pulmonary toxicity of 3-methylfuran, an atmospheric contaminant

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	M.R. Boyd	Research Associate	LCP NHLI
	R.B. Franklin	Visiting Fellow	LCP NHLI
	J.R. Mitchell	Chief, Sec. on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

We have found that 3-methylfuran is highly toxic to the pulmonary bronchiolar lining cells of experimental animals. Studies are underway to determine whether metabolic activation of this compound to a potent alkylating agent is the biochemical mechanism underlying the pathogenesis of these lesions, as we have shown for several other furano compounds. This finding may be of importance in human respiratory diseases because recent reports have shown that 3-methylfuran is a major air pollutant in cities such as Washington,D.C.

PHS-6040
(12-75)

Project Description:

Objectives: A recent report from the Naval Research Laboratory [Biomedical Mass Spectroscopy 1, 192 (1974)] indicated that a major atmospheric contaminant present during a smog alert in Washington, D.C., in August 1973, was 3-methylfuran (3MF). The authors speculated that 3MF is formed from atmospheric photooxidation and degradation of volatile hydrocarbons such as isoprene and terpenes. These naturally occurring precursors are produced in vast amounts and released into the atmosphere by vegetation. Deciduous forests, such as those covering the Appalachian Mountains, are said to exemplify a significant source. Presumably, under certain weather conditions, such as those that occurred in the Washington area in August 1973, these hydrocarbons may be confined in huge stagnant air masses. Oxidation of these substances may then produce concentrations of such products as 3MF, ozone, and other photochemical oxidants, which may result in a smog alert.

The Navy study aroused considerable interest and controversy both among the public and among scientists and several governmental agencies (e.g., Washington Post, June 10, 1975), particularly since it raised considerable question as to the relative contribution that automobile emissions make to certain urban smogs. Automobiles are widely considered to be the major source of atmospheric pollutants, especially in nonindustrial areas such as Washington, D.C.

The Navy study also stimulated our own interest in the toxicological properties of 3MF. In recent years we have found that many different furan derivatives produce necrosis of certain tissues of experimental animals when they are administered orally or parenterally. Necrosis results from the metabolism of the parent compounds to potent alkylating agents in the target tissues. One particularly intriguing example is that of 4-ipomeanol (IPO). IPO is a naturally-occurring furan derivative that is responsible for the severe and potentially fatal lung disease often seen in cattle which have been fed culled sweet potatoes [Biochem. Biophys. Acta 337, 184 (1974)]. IPO also produces lethal pulmonary lesions in all common laboratory animals when administered either orally or parenterally. The pulmonary toxicity of IPO is due to metabolism of the compound in the lung to a highly reactive metabolite which alkylates pulmonary tissue.

With this precedent, therefore, we are examining the metabolism and toxicological properties of 3MF in rats and mice.

Methods Employed: Standard methods are being employed.

Major Findings: 1) Preparation of 3-methylfuran. 3MF is not commercailly available. It was, therefore, necessary to develop a suitable method for synthesis of large quantities of 3MF as well as for preparation of small quantities of radiolabeled 3MF of high specific activity. Several approaches were explored. The procedure we have finally adopted requires 3-furoic acid (FA) as starting material. FA is reduced to the corresponding carbinol using a borane-methylsulfide complex in the presence of trimethylborate. The

alcohol is converted to the chloride with thionyl chloride and then dehalogenated to form 3MF using $LiAlH_4$. The overall yield of 3MF from FA is 40-50%. Specifically labeled 3MF (^3H) is presently being prepared using $LiAlH_4$ (^3H) in the final step of the above sequence. Purification of the final products is accomplished using a preparative gas chromatographic method we have developed.

2) Toxicity of 3-methylfuran in mice. NIH-GP mice were given 3MF intraperitoneally in a range of doses. Animals receiving doses of > 80 mg/kg, showed a striking necrosis of the pulmonary bronchiolar lining cells. At these doses, no lesions are seen in other tissues, using light microscopy. The bronchiolar lesions are indistinguishable from those produced by 4-ipomeanol.

3) Toxicity of 2-methylfuran in mice. 2-Methylfuran (2MF) (Aldrich) was also administered to NIH-GP mice to compare with 3MF. The 2-isomer similarly produces bronchiolar cell necrosis, but higher doses are required than for 3MF. 2MF appears to be less selective for the lung, since both renal and hepatic necrosis are also sometimes seen.

4) Apparatus for inhalation studies with 3MF. Glass metabolism cages have been modified for use in inhalation toxicity studies with 3MF. The 3MF is volatilized by slow infusion (Hamilton infusion pump) into a heated glass chamber which is continuously purged with a carefully metered flow of pure air. The effluent of the volatilization chamber is passed into the animal chamber. Provision has been made for careful monitoring of both the inflow and outflow rates as well as for removing gas samples from the animal chamber. A method has been devised, using gas chromatography, for the rapid quantitative analysis of 3MF in gas samples. Preliminary studies indicate that very stable and reproducible concentrations of 3MF can be produced in the chamber atmosphere. The exposure dose can therefore be easily varied by altering either the exposure time or the atmospheric concentration of 3MF.

Significance to Biomedical Research and to the Program of the Institute: We have found that, like certain other furan derivatives, 3MF is highly toxic to bronchiolar lining cells of the lung. It is probable that the 3MF-induced lesions, like those produced by 4-ipomeanol, result from pulmonary metabolism of the parent compound to form a potent alkylating agent. These observations, in addition to the recent report that 3MF may be a major atmospheric contaminant under certain conditions, indicate that 3MF should be further evaluated as a possible factor in the pathogenesis of chronic lung disease in man, such as bronchitis, fibrosis, emphysema, and cancer.

Proposed Course of Project: Studies are underway to further characterize both the acute and chronic pathological changes produced by 3MF. Morphological studies will involve light microscopy and both scanning- and transmission-electron microscopy; autoradiographic studies will be done using ^3H-3MF at both the light and EM levels. Studies will be done using both the ip and inhalation modes of administration. We are also continuing to examine the biochemical basis for the production of lung lesions by 3MF and related

furans with a view towards identifying factors which may regulate susceptibility to their toxic actions.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00900-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Hepatic and Renal Necrosis induced by 2-substituted Furans and Thiophenes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.J. McMurtry	Clinical Associate	LCP NHLI
OTHER:	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Recent studies from this laboratory have demonstrated that the massive hepatic necrosis in mice produced by the diuretic furosemide is mediated by the generation of reactive metabolites apparently derived from the 2-furyl methyl moiety of the molecule. Because of this finding and the close structural similarity of the thiophene residue contained in the nephrotoxic cephalosporins, cephaloridine and cephalothin, we have investigated the toxicology of furan and thiophene structural analogs. Simple 2-substituted furans and thiophenes induce a variety of tissue lesions in experimental animals including hepatic necrosis, renal tubular necrosis or combined hepatic and renal damage. Many, but not all, of these compounds also concomitantly deplete target organ glutathione. Alterations in target organ necrosis and glutathione depletion in pretreated animals implicate metabolic activations in the pathogenesis of those lesions. No correlation is apparent between target organ specificity, necrosis, or glutathione depletion and the chemical nature of the 2-substituent.

3/3

PHS-6040
(12-75)

Project Description:

Objectives: Recent work from this laboratory has demonstrated that the widely used diuretic, furosemide, undergoes metabolic conversion by mouse hepatic microsomal enzymes to a reactive arylating metabolite that produces massive hepatic necrosis in mice. This hepatic injury apparently results from the metabolic activation of the furan ring of furosemide N-(2'-furylmethyl)-4-chloro-5-sulphamoylanthranilic acid . Our studies also suggest that the dose-related nephrotoxicity induced by the cephalosporin antibiotic cephaloridine, (N- 7-(2'-thienylacetamidoceph-3-ylmethyl) pyridinium-2-carboxylate) may also be mediated by an active metabolite. A 2-substituted thiophene, an analog of furan in which the oxygen atom in the five-membered ring is replaced with sulfur, is present in both cephaloridine and the nephrotoxic cephalosporin, cephalothin 7-(2-thienyl acetamido) cephalosporanic acid , and may be involved in the pathogenesis of the tissue lesions produced by these compounds.

To elucidate further the pathogenesis of these lesions, the parameters influencing target organ specificity and the dose threshold, we have investigated the histologic changes and glutathione depletion in liver and kidney induced by a series of 2-substituted furans and tiophenes.

Methods Employed: Furan, 2-furamide, 2-acetyl furan, 2-methyl furan, 2-ethyl furan, 2-furfurol, 2-furoic acid, 2-ethyl furoate, 2,3-benzofuran, 2-furaldehyde, 2-methoxyfuran, thiophene, 2-ethyl thiophene, 2-thenoic acid, and 2-thienyl acetic acid were obtained from commercial sources. 2-Thenamide, 2-thienylacetamide, 2-ethylthenoate, and 2-thienylacetic acid ethylester were prepared from the corresponding acids by formation of the acyl chloride with thionyl chloride/toluene and subsequent reaction with anhydrous ammonia or ethyl alcohol, respectively.

Animal Studies: Male NIH GP mice (20-25g) received a single intraperitoneal injection of the 2-substituted furan or thiophene in sesame oil. The animals were pretreatmented as follows: mice--1) phenobarbital (80 mg/kg, i.p. daily for 3 days), 2) cobaltous chloride (30 mg/kg i.p. twice daily for 2 days), 3) peperonyl butoxide (1360 mg/kg i.p., 60 min. prior to injection), or 4) diethyl maleate (300 mg/kg, 1:3 in sesame oil i.p., 60 min. prior to injection). Animals were sacrificed at 36 hours and hepatic and renal tissues processed and scored for histologic damage according to previously described methods. Hepatic and renal glutathione levels were determined at multiple time intervals at the injection of the furan (or thiophene) by previously described analytical methods. Animals were pretreated, as detailed above.

Major Findings: A spectrum of hepatic and renal lesions were induced in NIH GP mice by a single, sublethal, i.p. injection of 2-substituted furans or thiophenes. (Table I) Thiophene, 2-acetyl furan and 2-furfurol were primarily hepatotoxins. Furan, 2-ethyl furan, and 2-furamide exhibited both hepatic and renal lesions while 2,3-benzofuran, 2-furoic acid, 2-thenamide, and 2-ethylthenoate were primarily nephrotoxins. 2-Methyl furan, 2-methoxy furan, and 2-thienylacetic acid failed to induce either hepatic or renal histologic lesions even at doses exceeding 500 mg/kg in mice. Marked tissue

glutathione depletion was almost invariably accompanied by concomitant tissue necrosis. (Table I) Conversely, 2-substituted furans capable of inducing hepatic necrosis also depleted hepatic glutathione. However, a less predictable relationship was observed between renal necrosis and renal glutathione depletion. Although, renal necrosis paralled renal glutathione depletion with furan,2-ethyl furan and 2-furamide, several nephrotoxins including 2-furoic acid and 2,3-benzofuran had minimal if any effect on target organ glutathione levels. Also, a moderate depletion of renal GSH by 2-acetyl furan and thiophene did not result in renal necrosis.

Both necrosis and glutathione depletion were generally decreased by pretreatment of animals with cobalt chloride or piperonyl butoxide. Pretreatment with phenobarbital, an inducer of multiple hepatic enzymatic pathways but not renal cytochrome P-450 oxidases, had a variable effect on liver histology and glutathione depletion but invariably either decreased or had no effect on the corresponding renal parameters.

Several compounds including the potent nephrotoxin, 2,3-benzofuran, had little effect on target organ glutathione. Since the tissue injury produced by these compounds presumably results from the metabolic activation of the furan ring, failure to deplete tissue glutathione may reslut from the inability of the reactive intermediate species derived from 2,3-benzofuran and certain other compounds to act as substrates for the enzyme glutathione-S-epoxide transferase. The inability of furosemide and cephaloridine to deplete liver and renal glutathione, respectively, may also reflect this phenomena. No apparent correlation could be demonstrated between chemical structure (furyl-substituent effect) and target organ preference, target organ glutathione depletion, target organ tissue necrosis, or acute toxicity (LD_{50}) of the compound.

Significance to Biomedical Research and the Program of the Institute:

A number of simple 2-substituted furans and thiophenes that are structural analogs of furosemide and cephaloridine, have been shown to produce a variety of tissue lesions including hepatic necrosis, renal tubular necrosis, or combined hepatic and renal damage in experimental animals. Concomitant target organ glutathione depletion occurs with many of these compounds. Both target organ necrosis and glutathione depletion, when present, appear to result from a metabolic activation similar to that previously proposed for the hepatotoxins furosemide, acetaminophen and isoniazid.

Proposed Course of Project: Completed.

Publications: None.

Table 1: Hepatic and renal necrosis and glutathione depletion induced in mice by 2-substituted furans and thiophenes

Compound	LD_{50}(mg/kg)	histology* dose(mg/kg)	liver	kidney	GSH depletion‡ dose(mg/kg)	liver	kidney
2-methoxy-furan	800	780	0	0	N.A.	N.A.	N.A.
2-furaldehyde	150	140	0	0	250	0	+
2-methylfuran	N.A.	600	0	0	N.A.	N.A.	N.A.
2-acetylfuran	125	80	+	0	80	++	0
		115	+++	0	115	++++	++
2-furfurol	300-400	225	++	0/+	250	++	+
2-ethyl-furoate	750	400	0	+			
		700	+++	0	700	+++	++
furan	360	200	+/++	0/+	225	++	0
		340	++	++	300	++++	++++
2-ethylfuran	250	200	+	++	200	++++	++++
2-furamide	N.A.	115	++	0	125	++	0
		300	++++	++	250	+++	++
2-furoic acid	200	175	0	+/++	175	+	+
2,3-benzo-furan	175	100	0	++	100	0	0
		200	0	++++	N.A.	N.A.	N.A.
Thiophene	500-600	250	++/+++	0	250	++++	++
cephaleridine	N.A.	3000	0	++	3000	++	0
-thienyl-acetic acid	N.A.	500	0	0	N.A.	N.A.	N.A.
-thienyl-acetamide	N.A.	500	0	0/+	N.A.	N.A.	N.A.
2-thenamide	N.A.	100	0	+	N.A.	N.A.	N.A.
2-ethyl thenoate	N.A.	100	0	+	N.A.	N.A.	N.A.

‡ GSH depletion = minimum value recorded, as 0/0 of control
80-100 = 0; 50-80 = 1+; 40-60 = 2+; 25-40 = 3+; 0-25 = 4+.

* scored for histologic damage according to previously described methods.

N.A. = Not Attempt

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00901-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cephalosporin Nephrotoxicity: Possible Role of Metabolic Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.J. McMurtry	Clinical Associate	LCP NHLI
OTHER:	J.R. Mitchell	Chief, Section on Clin. Pharm.	LCP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Dose dependent cephaloridine-induced renal tubular necrosis was observed in mice, rats, and rabbits following a single i.p. injection of cephaloridine. Necrosis was prevented, but nor reversed, by prior treatment with inhibitors of drug metabolism such as piperonyl butoxide and cobaltous chloride, suggesting that the nephrotoxicity of this cephalosporin antibiotic may be mediated by metabolic activation to a chemically reactive species. Necrosis was potentiated by the concomitant administration of aminoglycoside antibiotics and by diethyl maleate, a depletor of hepatic and renal glutathione. Experimental attempts to demonstrate covalent binding of cephaloridine metabolites to renal proteins in vivo and renal microsomal protein in vitro were rendered uninterpretable by facile exchange of label from the radioisotope employed. Studies are in progress to define further the role of metabolic activation in cephalosporin-induced nephrotoxicity and the mechanistic basis for aminoglycoside enhancement of toxicity.

PHS-6040
(12-75)

Project Description:

Objectives: Recent studies from this laboratory have demonstrated that the massive hepatic necrosis in mice produced by the diuretic furosemide is mediated by the generation of reactive metabolites apparently derived from the 2-furyl methyl moiety of this molecule. Because of the close structural similarity of the thiophene residue contained in the nephrotoxic cephalosporins, cephaloridine and cephalothin, we have initiated a study of the pathogenesis of nephrotoxicity induced by these drugs.

Methods Employed: Male NIH GP mice (20-25 g), male Sprague-Dawley rats (180-200 g), Male Fischer rats (180-200 g) or male white New Zealand rabbits (2kg) received a single intraperitoneal injection of cephalosporin in normal saline. Pretreatments of mice and rats with cobaltous chloride, piperonyl butoxide, phenobarbital or diethyl maleate were carried out as previously described. Additional pretreatments in Sprague-Dawley (S-D) and Fischer (F) rats were as follows: kanamycin (500 mg/kg, i.p., S-D), gentamicin (100 mg/kg, i.p., F; 200 mg/kg i.p., S-D), and neomycin (100 mg/kg i.p., F; 200 mg/kg i.p., S-D). Pretreatment of rabbits was as follows: phenobarbital (125 mg/kg daily for 7 days, p.o. in drinking water, then tap water for 1 day prior to injection). Animals were sacrificed at 24, 36 or 48 hours with tissues processed and graded for histologic damage according to previously detailed techniques.

Cephaloridine (Loridine, Eli Lilly) and sodium cephalothin (Keflin, Eli Lilly) were obtained from commercial sources and used without additional purification. α-Thienylacetyl-^{3}H labeled cephalothin (I) and cephaloridine (II) were synthesized according to procedures detailed in the NIH Progress Report: Synthesis of Radiolabeled Cephalosporins.

In vivo covalent binding studies: Animals received a single i.p. dose of cephalosporin and were sacrificed at 3 hours post-injection. Portions of liver, kidney and sartorius muscle were excised, homogenized, and metabolite binding determined by previously detailed procedures employed in this laboratory.

In vitro covalent binding studies: Washed liver and renal microsomes were prepared and the irreversible binding of metabolites to protein determined as previously described for other compounds.

Major Findings: Following a single i.p. injection of cephaloridine, dose-dependent cephaloridine-induced renal tubular necrosis was observed in mice (3000 mg/kg), rats (1000-2000 mg/kg), and rabbits (200 mg/kg). Necrosis was prevented, but not reversed, by prior treatment with inhibitors of drug metabolism such as piperonyl butoxide and cobaltous chloride. Animals pretreated with phenobarbital, an inducer of hepatic but not renal cytochrome P-450 enzymatic pathways in rats and mice, had no discernable effect on renal necrosis. Phenobarbital pretreatment likewise failed to alter significantly the severity of cephaloridine-induced renal necrosis in NZ rabbits, although induction of renal as well as hepatic enzymes have been reported in this species. Apparent irreversible binding of radiolabeled cephaloridine

(metabolite) to target organ tissues, predominantly kidney, occurred following administration of α-thienylacetyl-^{3}H labeled cephaloridine to rats or mice. However, the (apparent) binding levels were unaltered by pretreatments having profound effect on histological necrosis. Incubation of radiolabeled cephaloridine with rat renal microsomes resulted in exceedingly high levels of noncofactor dependent binding. These anomalous findings may result from a facile exchange of tritium label in the α-thienylacetyl portion of cephaloridine.

A protective effect of probenecid and other organic anions against cephaloridine nephrotoxicity was reported by Child and Dodds (Child, K.J., and Dodds, M.G., "Nephron transport and renal tubular effects of cephaloridine in animals". Br. J. Pharmac. Chem., 30, pp. 354-370, (1967).). More recent work by Tune and co-workers (Tune, B.M. et. al., "Mechanism of cephaloridine transport in the kidney". J. Pharmac. Exp. Thera., 191, (1974), pp. 311-317.) has established that cephaloridine must be actively concentrated in the proximal tubular cell to exert a nephrotoxic effect. Preliminary experiments suggest that the renal uptake of drug is not significantly altered by cobaltous chloride pretreatment associated with protection against tissue lesions. To determine whether concurrent administration of an aminoglycoside would enhance the nephrotoxic potential of a cephalosporin, rats were given a single dose of cephaloridine and either gentamicin, kanamycin or neomycin. Enhanced nephrotoxicity occured in most instances.

Significance to Biomedical Research and the Program of the Institute: Cephaloridine (Loridine, Eli Lilly and Co.) and sodium cephalothin (Keflin, Eli Lilly and Co.) are semi-synthetic cephalosporin antibiotics which can produce nephrotoxicity in man. The dose-related nephrotoxic lesion in experimental animals can be prevented by prior treatment with inhibitors of drug metabolizing enzymes such as piperonyl butoxide or cobaltous chloride, suggesting that the nephrotoxicity of these commonly employed antibiotics may be mediated by metabolic conversion to reactive intermediate species. Additionally, cephaloridine nephrotoxicity in rats is clearly potentiated by concomitant injection of aminoglycoside antibiotics, which may be mechanistically analagous to the clinically apparent nephrotoxic synergism of gentamicin-cephalothin regimens in man.

Proposed Course of Project: Possible in vivo and in vitro transformation of cephalosporins to reactive chemical species will be examined upon the completion of cephaloridine-^{14}C and cephalothin-^{14}C synthesis. Aminoglycoside enhancement of cephalosporin nephrotoxicity will be investigated with regard to altered cephalosporin toxifying/detoxifying mechanisms.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00902-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Role of Metabolic Activation in 2-Furamide-induced Renal and Hepatic Necrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.J. McMurtry	Clinical Associate	LCP NHLI
Other:	J.R. Mitchell	Chief, Sec. on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.4

SUMMARY OF WORK (200 words or less - underline keywords)

2-Furamide, a structural analog of the diuretic furosemide, produces centrilobular liver necrosis and renal tubular necrosis in mice. Pretreatment with inhibitors of metabolism decreased irreversible binding of metabolite and glutathione depletion in target organ tissues while concomitantly protecting against histologic tissue damage. Induction with phenobarbital tended to enhance hepatic necrosis, glutathione depletion and covalent binding while having little effect on the corresponding renal parameters. Covalent binding of radiolabeled material to mouse renal or hepatic microsomes was dependent on NADPH, oxygen and temperature and was inhibited by a carbon monoxide-oxygen atmosphere. These experiments demonstrate that 2-furamide undergoes in vivo and in vitro activation in the mouse liver and kidney to reactive species producing hepatic and renal necrosis. The differential in vivo target organ response to phenobarbital induction and in vitro metabolite binding studies suggest that renal necrosis results from in situ metabolic activation rather than hepatic metabolism. Thus, the spectrum of hepatic and renal lesions induced in experimental animals by simple 2-substituted furans and thiophenes as well as more complex structural analogs may result from organ specific metabolic activation.

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Project Description:

Objectives: The diuretic, furosemide, has recently been noted to produce massive hepatic and renal necrosis in mice. Covalent binding with target organ macromolecules in the liver, is apparently mediated by the formation of a chemically reactive furosemide metabolite in the liver. Structural analogs of furosemide containing a 2-substituted furan ring have been found to cause a variety of lesions in experimental animals including renal tubular necrosis, hepatic necrosis, and combined renal and hepatic damage. Alterations in target organ toxicity in pretreated animals suggest that these tissue lesions may result from a metabolic activation similar to that proposed for furosemide. Because of these findings, we elected to examine the hepatic and renal lesions in mice induced by the furosemide analog, 2-furamide.

Primary objectives were: i) to determine whether those lesions were mediated by reactive metabolites, ii) to determine whether the respective target organs were also the sites of metabolic activation, and iii) to examine factors influencing the relative severity of the different target organ lesions.

Methods Employed: 2-Furamide was obtained from Mallinckrodt. Randomly labeled 2-furamide-^{3}H was obtained via catalytic tritium exchange of 2-furamide (New England Nuclear Co.).

Histologic studies: Male NIH GP mice (20-25 g) received a single intraperitoneal injection of furamide with pretreatments as follows: cobaltous chloride (30 mg/kg ip twice daily for 2 days), phenobarbital (75 mg/kg ip, daily for 3 days), piperonyl butoxide (1360 mg/kg, ip, 30 min prior to injection), or diethyl maleate (600 mg/kg, ip, 60 min prior to injection). Animals were sacrificed at 36 hr with tissues processed and scored for histologic damage according to previously described techniques.

Glutathione depletion studies: Animals received a single injection of furamide, with pretreatments as described above. Following sacrifice at varying time intervals after injection of furamide, tissue glutathione concentrations were determined by previously described assay procedures.

In vivo covalent binding studies: Animals received a single ip dose of 2-furamide and were sacrificed at 4 hr after injection. Portions of liver, kidney and sartorius muscle were excised, homogenized, and metabolite binding determined by previously detailed procedures employed in this laboratory.

In vitro covalent binding studies: Washed liver and renal microsomes were prepared and incubated under various conditions with different concentrations of 2-furamide-^{3}H and irreversible binding of metabolites to protein determined as previously described for other compounds.

Major Findings: 2-Furamide produced only centrilobular liver necrosis in mice at doses of 100-150 mg/kg ip but both centrilobular liver necrosis and acute renal tubular necrosis at higher doses (200-300 mg/kg). 2-Furamide (250 mg/kg ip) also depleted hepatic glutathione by over 70% within 120-180 min after injection. A similar, but quantitatively less marked, depletion of renal glutathione was also noted.

A dose-dependent irreversible binding of furamide (metabolite) to target organ proteins occurred in both liver and kidney. Pretreatment of animals with inhibitors of metabolism, such as cobaltous chloride and piperonyl butoxide, decreased the severity of hepatic and renal necrosis and the extent of target organ tissue binding of 2-furamide-^{3}H metabolites. Pretreatment of animals with phenobarbital, an inducer of drug metabolism, appeared to enhance hepatic necrosis at doses of 100-115 mg/kg of 2-furamide with no discernable histologic effect on the kidney. Covalent binding of metabolite to hepatic protein was increased after phenobarbital but binding to renal protein was moderately decreased.

Target organ glutathione depletion by 2-furamide was decreased in animals pretreated with cobaltous chloride or piperonyl butoxide whereas phenobarbital enhanced hepatic glutathione depletion without significantly affecting renal glutathione levels.

Washed hepatic or renal microsomes were incubated with 2-furamide-^{3}H under various conditions. Covalent binding of radiolabeled material to mouse liver or renal microsomes was dependent on NADPH and oxygen. The binding to both renal and hepatic microsomes was inhibited by a 90:10 carbon monoxide: oxygen atmosphere.

Significance to Biomedical Research and the Program of the Institute: The compound 2-furamide has been shown to undergo *in vivo* and *in vitro* activation in the mouse liver and kidney to a reactive species producing hepatic and renal necrosis. *In vitro* metabolite binding studies and the differential target organ response to phenobarbital induction suggest that renal necrosis results from in situ metabolic activation rather than hepatic metabolism. Thus, the spectrum of hepatic and renal lesions induced by simple 2-substituted furans and thiophenes as well as more complex structural analogs such as cephalothin and cephaloridines may result from organ specific metabolic activation.

Proposed Course of Project: Completed.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00903-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Xenobiotic-altered patterns of histologic response to nephrotoxin-induced renal injury

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	R.J. McMurtry	Clinical Assoc.	LCP NHLI
	J.R. Mitchell	Chief, Sec. on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

SUMMARY OF WORK (200 words or less - underline keywords)

Altered severity of necrosis or a shift in location of certain drug-induced renal cortical lesions have been observed following pretreatment with sulfhydryl-containing or glutathione-depleting compounds. Cephaloridine-induced outer cortical necrosis undergoes a striking shift to inner cortical necrosis in animals pretreated with cysteine or DTT; the histologic changes are species or strain dependent. These observations implicate an as yet undefined role for sulfhydryl-containing compounds in the pathogenesis of drug-induced renal disease. The histologic shift in necrosis may result from selection of a different population of nephrons (juxtamedullary rather than cortical) or from a shift to a more distal portion (pars recta) of the same nephron population. We propose to resolve this question by microdissection, enzyme histochemistry and/or urinary enzyme assay and to extend our investigations to nephrotoxic lesions of other pathogenesis such as $HgCl_2$ lesions.

PHS-6040
(12-75)

Project No. Z01 HL 00903-01 LCP

Project Description:

Objectives: Although altered patterns of toxin-induced histologic tissue damage have occasionally been noted in animals induced with a second chemical, little is known concerning the exact morphological or biochemical basis for these changes. We have previously reported that pretreatment with phenobarbital shifts the zone of hepatic necrosis produced by furosemide from a midzonal to centrilobular distribution in mice. Similar observations have been reported with other furan containing compounds, such as ngaione and 3-hydroxymethylfuran. This shift in the zone of furan-induced hepatic injury after phenobarbital pretreatment may result from differences in the degree of enzyme induction of toxifying and detoxifying pathways in centrilobular versus midzonal hepatocytes. These observations have prompted us to examine analogous alterations of renal histologic damage in attempting to further define nephrotoxin-kidney interactions.

Methods Employed: Male Fischer rats (180-200g) or male Sprague-Dawley rats (180-200g) received a single ip dose of a nephrotoxin with pretreatments as follows: (rats) cobaltous chloride (30 mg/kg sc twice daily for 2 days); piperonyl butoxide (600 mg/kg cp); phenobarbital (75 mg/kg daily for 3 days); diethyl maleate (450-600 mg/kg ip 60 min before use); cysteamine (150 mg/kg sc, 5 min before use); dithiothreitol (DTT) (30 mg/kg ip, 90 and 15 min prior to use); and cysteine (150 mg/kg sq 5 min before and 20 min after use). Animals were sacrificed at 24 hr and tissues processed and graded for severity of histologic damage according to previously described techniques.

Major Findings: Pretreatment of experimental animals with cytochrome P-450 enzyme inhibitors afforded protection against a variety of nephrotoxins, including simple 2-substituted furans and thiophenes, cephaloridine, p-hydroxyacetanilide, and p-aminophenol. The pattern of histologic necrosis, although quantitatively reduced or eliminated, was not altered by pretreatment. These observations are consistent with the currently proposed role of renal metabolic activation in the pathogenesis of these lesions. However, pretreatments that altered the availability of glutathione or the administration of sulfhydryl-containing compounds shifted the location of histologic renal damage. These changes were often species or strain specific for the nephrotoxic antibiotic cephaloridine: pretreatment of mice with diethyl maleate to deplete renal glutathione increased and shifted the proximal tubular necrosis from outer cortex location to the mid-inner cortex. Similar pretreatment of either Sprague-Dawley or Fischer rats clearly potentiated the nephrotoxicity without clearly shifting its location. Pretreatment of Sprague-Dawley rats with cysteine resulted in a striking shift of necrosis from outer to inner cortex. Similar pretreatment of Fischer rats afforded more marked protection against histologic necrosis although residual necrosis appeared to have been shifted to inner cortex. A partial shift of the cephaloridine-induced tubular necrosis from outer to inner cortex was also observed following pretreatment with dithiothreitol. In contrast, cysteamine pretreatment afforded a high degree of protection against histologic renal damage in both strains of rats.

Significance to Biomedical Research and to the Program of the Institute: A striking shift in the location of certain drug-induced renal cortical lesions has been observed following pretreatment with sulfhydryl-containing or glutathione-depleting compounds. It is not known whether this represents an anatomical shift within the same population of nephrons, (shift from the pars convoluta to pars recta) or a shift to a different population of nephrons (cortical to juxtamedullary nephrons). These observations implicate an as yet undefined role for sulfhydryl-containing macromolecules in the pathogenesis of drug-induced renal disease.

Proposed Course of Project: Resolution of anatomical nature of the shift in necrosis will be attempted by both microdissection techniques and enzyme histochemistry and/or urinary enzyme assay for enzymes predominant in either the pars convoluta or pars recta of the proximal convoluted tubule.

Additionally, this apparent interaction between nephrotoxins and sulfhydryl containing compounds will be extended to include examples of compounds of differing suspected pathogenesis (direct toxicity such as $HgCl_2$ vs metabolic activation-mediated) which characteristically damage discrete areas within the renal cortex in the absence of the pretreatment we have used (e.g., $HgCl_2$, permanganate).

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00904-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Metabolic Activation of 1,4-Ipomeadiol to a Selective Nephrotoxin.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.J. McMurtry	Clinical Associate	LCP NHLI
OTHER:	M.R. Boyd	Research Associate	LCP NHLI
	J.R. Mitchell	Chief, Sec. on Clin. Pharm.	LCP NHLI

COOPERATING UNITS (if any)
Dr. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

SUMMARY OF WORK (200 words or less - underline keywords)

1,4-Ipomeadiol (I), a furanoterpenoid derived from mold-damaged sweet potatoes is a dose-dependent selective nephrotoxin in mice. Approximately 30 grams of (I) have been obtained along with precursor following multi-batch synthesis beginning with 3-ethyl furoate. Preliminary studies suggest that the metabolite of (I) is covalently bound to liver, lung and kidney macromolecules, with the highest binding levels occurring in the kidney. The relatively high specificity for renal tissue in the mouse suggests that 1,4-Ipomeadiol may be a valuable probe in the elucidation of drug-induced nephrotoxicity and studies are in progress to further define the metabolic basis for this organ-selective histologic damage and metabolite-binding. The relevance of these findings to the possible hazard in man resulting from the ingestion of small amounts of this coumpound is unknown at this time.

PHS-6040
(12-75)

Project Description:

Objectives: The furanoterpenoid, 1,4-ipomeadiol 1-(3-furyl)-1,4-pentanediol is one of a group of at least four closely related compounds constituting the "lung edema factor" produced by mold-damaged sweet potatoes. Mice surviving the pulmonary toxicity of near-lethal doses of 1,4-ipomeadiol have been observed to develop acute renal necrosis. Our previous work with simple 2-substituted furans has demonstrated that these compounds can undergo metabolic transformation in both liver and kidney to reactive, toxic species. Therefore, we have examined the possible role of metabolic activation in the pathogenesis of 1,4-ipomeadiol-induced renal damage.

Methods Employed: (a) Synthesis. The preparative scale synthesis of 1,4-ipomeadiol was carried out according to methods of Boyd involving the initial condensation of 3-ethyl furoate with ethyl acetate followed by reaction with propylene oxide in base. The lactone thus formed was decarboxylated by gentle heating in dilute acid and the resulting 4-ipomeanol reduced to 1,4-ipomeadiol with lithium aluminum hydride.

(b) Screening toxicity and *in vivo* covalent binding studies. NIH Swiss GP mice received a single intraperitoneal dose of low specific activity ^{14}C-1,4-ipomeadiol in 25% propylene glycol/H_2O. Animals were sacrificed at 24 hours. One kidney and a small portion of the liver were removed for histologic examination; the remaining liver contralateral kidney, lung and guandraceps muscle were removed and covalent binding of reactive intermediate to tissues was determined according to previously described methods.

Major Findings: Ethyl 3-furoyl acetate (ca. 157g) was prepared from the (multi-batch) Claissen condensation of 3-ethyl furoate and ethyl acetate with about a 45% yield. 4-Ipomeanol (43.8g) was then synthesized from 74.0g of ethyl 3-furoyl acetate via the intermediate, 2-(3-furoyl)-4-hydroxy pentanoic acid, -lactone. Crude 1,4-ipomeadiol (34.2g) was obtained by reduction of the 4-Ipomeanol with lithium aluminum hydride.

Preliminary toxicology studies show 1,4-ipomeadiol to be a dose-dependent, selective nephrotoxin in mice resulting in extensive acute tubular necrosis at a dose of 80 mg/kg. Covalent binding of reactive intermediate to mouse kidney and lung tissue occurs with the highest level of binding occurring in the kidney over the dose range (20-80 mg/kg) examined. Less covalent binding to kidney was observed in the rat along with a relative increase in binding to pulmonary tissue. These findings, the absence of renal histological necrosis and the high mortality of rats receiving doses comparable to those resulting in renal necrosis in mice, are consistent with predominant pulmonary toxicity in the Fischer rat.

Significance to Biomedical Research and Progress of the Institute: Preliminary evidence suggests that 1,4-ipomeadiol is metabolized by hepatic and extra-hepatic tissues to a reactive intermediate species. The relatively high specificity for renal tissue in the mouse suggests that 1,4-ipomeadiol may be a valuable model compound in the further elucidation of toxification-detoxification interactions involving the kidney and xenobiotics.

The possible significance of these pathologic findings to the ingestion of mold-damaged sweet potatoes by man and other animals is uncertain at the present time.

Proposed Course of Project:

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00905-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Synthesis of Radiolabeled Cephalosporins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.J. McMurtry	Clinical Associate	LCP NHLI
OTHER:	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The cephalosporin antibiotics cephalothin and cephaloridine can produce nephrotoxicity in man. Ongoing studies in this laboratory concerned with the molecular pharmacology of this nephrotoxicity have required the use of selectively labeled cephalothin and cephaloridine. We have successfully prepared cephalothin-^{3}H and cephaloridine-^{3}H with randomly labeled α-thienylacetic acid. However, because of the facile exchange of tritium with these compounds, the corresponding cephalothin-^{14}C and cephaloridine-^{14}C are currently being synthesized from α-thienyl acetic acid-1-^{14}C. A small quantity of cephaloridine-(2,6-pyridine-^{14}C), has also been prepared.

PHS-6040
(12-75)

Project Description:

Objectives: Cephaloridine (Loridine, Eli Lilly and Co.) and cephalothin (Keflin, Eli Lilly and Co.) are semisynthetic cephalosporin antibiotics which can produce nephrotoxicity in man. Recent studies from this laboratory suggest that the dose-related nephrotoxicity of cephaloridine in experimental animals may result from the metabolic transformation of the parent drug. Possible covalent binding of drug metabolite to tissue macromolecules can most readily be evaluated utilizing radiolabeled parent compound. Because of the unavailability of radiolabeled cephalosporins from commercial sources, we have undertaken the synthesis of selectively labeled cephalothin and cephaloridine.

Methods Employed: General catalytic tritium exchange of cephaloridine was attempted by New England Nuclear. Selective ^{3}H-labelling of the α-thienyl acetyl moiety of cephalothin and cephaloridine was attempted by first subjecting α-thienyl acetic acid (Marstan Chemical Co.) to non-specific catalytic tritium exchange (New England Nuclear). The corresponding acid chloride was obtained from the acid and thienyl chloride in toluene and subsequently coupled with 7-amino cephalosporonic acid (Eli Lilly and Co.). Optimal conversion was obtained utilizing a modified Schotten-Bauman's method with sodium bicarbonate in aqueous acetone. Crude cephalothin thus obtained could/be further purified by thin layer chromatography or reacted directly with pyridine in the presence of thiocyanate ion to yield the pyridinium betaine, cephaloridine, as described by Spencer (Spencer, J.L., Siu, F.Y., Jackson, B.G., Higgins, H.M. and Flynn, E.H., "Chemistry of Cephalosporin Antibiotics. IX. Synthesis of Cephaloridine". J. Org. Chem. 32, (1967), pp. 500-501). After treatment of the crude reaction products with an anion exchange resin (AG-1, formate form), cephaloridine was isolated by preparative scale thin layer chromatography (Avicel-F1000, 85 :15 acetone/water). Pyridine-2,6-^{14}C labeled cephaloridine was prepared from the reaction of cephalothin and pyridine-2,6-^{14}C (Amersham/Searle) with purification as described above.

Major Findings: Catalytic tritium exchange of cephaloridine did not prove to be a satisfactory technique for the preparation of radiolabeled cephaloridine. α-Thienyl acetyl-^{3}H labeled cephalothin (I) and cephaloridine (II) were conveniently synthesized via a sequence of reactions beginning with general catalytic-exchange labeled ^{3}H-α-thienyl acetic acid.

Pyridine-2,6-^{14}C-cephaloridine (IIa) was prepared from cephalothin and pyridine-2,6-^{14}C.

The facile exchange of tritium from (I) has precluded its use in covalent binding studies and we are currently attempting to synthesize the cephaloridine-(α-thienylacetyl-1-^{14}C). Completion of this synthesis is awaiting successful preparation of the ^{14}C-carboxylation of thenyl magnesium chloride by American Radiochemical Co.

Significance to Biomedical Research and the Progress of the Institute: Successful synthesis of the radiolabeled cephalosporins, cephalothin and cephaloridine will facilitate further illucidation of the nephrotoxicity associated with these compounds. The facile exchange of tritium atoms

incorporated in the thienyl moiety of these cephalosporins has been found to preclude their use in this application. Thus, we are currently attempting to synthesize the corresponding ^{14}C-labeled compounds for use in these studies.

Proposed Course of Project: Cephaloridine-(α-thienylacetyl-1-^{14}C) will be synthesized following successful synthesis and delivery of α-thienylacetic acid-^{14}C-(carbonyl) by American Radiochemical Co.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00906-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Metabolic Activation of Phenacetin to a Hepatotoxic Metabolite

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	W. Snodgrass	Guest Worker	LCP NHLI
Other	R.J. McMurtry	Clinical Assoc.	LCP NHLI
	J.R. Mitchell	Chief, Sec. on Clin. Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Snodgrass holds an NIH Research Fellowship with the National Inst. of Arthritis, Metabolism and Digestive Diseases.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

The minor analgesic, phenacetin, induces severe hepatic necrosis in 3-MC-treated hamsters. Antecedent hepatic glutathione depletion and tissue necrosis appear to be mediated by the metabolic conversion of phenacetin to a chemically reactive species capable of irreversibly binding to target organ protein. Because of the suspected contribution of phenacetin to the pathogenesis of analgesic nephropathy, we have attempted to extrapolate those findings to the kidney but have been unsuccessful thus far in developing an experimental model for phenacetin-induced renal damage.

PHS-6040
(12-75)

Project Description:

Objectives: The minor analgesic, phenacetin (p-ethoxyacetanilide) has been implicated in the pathogenesis of analgesic nephropathy. The major metabolites of phenacetin in man are the glucuronide and sulfate conjugates of acetaminophen (p-hydroxyacetanilide), another widely used minor analgesic. Previous studies from this laboratory have demonstrated that acetaminophen can be metabolized by hepatic and renal mixed-function oxidases to a reactive electrophilic species capable of binding to tissue macromolecules resulting in hepatocellular damage and renal tubular necrosis. We have, therefore, investigated the possibility that activation of phenacetin or its metabolites by target organ tissue might occur in a similar manner.

Methods Employed: Radiolabeled phenacetin-^{3}H was obtained via general catalytic tritium exchange of phenacetin by New England Nuclear. Phenacetin-^{14}C-carbonyl was prepared from p-ethoxyaniline and acetylchloride-1-^{14}C (New England Nuclear) by methods described in the literature (Vogel). p-Ethoxy-1-^{14}C) acetanilide was prepared from p-hydroxyacetanilide and ethyl iodide-1-^{14}C (Amersham/Searle) according to the method of Vogel.

Animals received a single ip or sc injection of phenacetin with pretreatment as follows: Syrian Golden hamsters, male, 70-80g), diethyl maleate (600-1200 mg/kg sq, 2 hr before use), 3-methylcholanthrene (3-MC) (20 mg/kg, ip, every 12 hr for 2 doses, then 24 hr sans treatment prior to use), piperonyl butoxide (1600 mg/kg, 30 min prior to use), cobaltous chloride (30 mg/kg sc, twice daily for 2 days). Male C57 B/6 mice, 20-25 g or male NIH GP mice, 20 g: diethyl maleate (1,000 mg/kg sc 2 hr prior to use), or 3-MC (20 mg/kg ip, daily for 2 days). Male Sprague-Dawley (S-D) or Fischer (F) rats: diethyl maleate (1,000 mg/kg sc, 2 hr prior to use or 300 mg/kg sc 30 min prior to use S-D), 600 mg/kg sq, 2 hr prior to use (F), 3-MC; (20 mg/kg ip, daily for 2 days (S-D).

Histologic studies were performed in animals sacrificed at 24 or 36 hr with tissue processed and graded for severity of necrosis according to previously described techniques from this laboratory. Tissue glutathione concentrations were also determined according to previously described procedures as were levels of irreversible metabolite binding in animals sacrificed at 1 and 3 hr post injection of radiolabeled phenacetin.

Major Findings: Phenacetin administered to Sprague-Dawley rats in sc doses of 600, 900 and 1200 mg/kg did not significantly deplete renal or hepatic glutathione or induce histologic tissue lesions. Similar results were obtained in the Fischer rat.

Phenacetin 900-1200 mg/kg sc depleted renal and hepatic glutathione levels to Ca 60% of control levels in NIH GP mice and resulted in occasional histologic evidence of centrilobular liver necrosis.

Hepatic glutathione levels were reduced to Ca 60% of control levels in hamsters receiving phenacetin 400 mg/kg or 1200 mg/kg, sc. However, in

animals pretreated with 3-MC, a striking decrease of hepatic glutathione was observed even with much lower doses of phenacetin, with concomitant severe and extensive hepatic necrosis. Regardless of route of administration or pretreatment there was little effect on renal glutathione and no histologic renal damage was observed.

In contrast to results with 3-MC pretreatment, hamsters pretreated with phenobarbital exhibited minimal, if any, enhancement of glutathione depletion. This latter finding may reflect the more nonspecific inducement of detoxifying and toxifying pathways with phenobarbital relative to 3-MC.

A dose-dependent irreversible binding of phenacetin to hepatic and renal proteins, predominantly the former, occurred in hamsters. Pretreatments (phenobarbital, cobalt chloride, piperonyl butoxide, diethyl maleate, methylcholanthrene) that altered phenacetin hepatic injury caused parallel changes in covalent binding of metabolite.

Significance to Biomedical Research and to the Program of the Institute: The minor analgesic, phenacetin has been shown to produce severe hepatic necrosis in the hamster at doses depleting hepatic glutathione stores. Hepatic glutathione depletion and tissue necrosis appear to be mediated by a metabolic activation of phenacetin, possibly after conversion to acetaminophen. Preliminary studies of irreversible metabolite binding with ethoxy-labeled phenacetin have been hampered by apparent incorporation of label into tissue protein via the acetate pool.

Proposed Course of Project: In vivo binding of selectively labeled phenacetin metabolite to hamster target organ proteins will be studied with concomitant cycloheximide inhibition of acetate incorporation. The search for a suitable animal model for renal toxicity will continue, utilizing differing mouse strains and augmented sc dosing of phenacetin.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00907-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Acetaminophen-Induced Nephrotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	W.R. Snodgrass	Guest Worker	LCP NHLI
OTHER:	R.J. McMurtry	Clinical Associate	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Snodgrass is on an NIH Fellowship 1 F32 AM 05360-01 sponsored by NIAMDD.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

Acetaminophen (p-hydroxyacetanilide) can result in acute renal damage in man when ingested in large amounts. Acetaminophen and its sulfate and glucuronide conjugates are also the major metabolites in man of phenacetin (p-ethoxyacetanilide), a compound implicated in the pathogenesis of analgesic nephropathy. Because our previous studies demonstrated that acetaminophen can be metabolized by the liver to a toxic intermediate species, we have investigated the possibility that extra-hepatic activation by the kidney might occur in a similar manner. Acetaminophen given subcutaneously was found to produce acute tubular necrosis in Fischer rats and to covalently bind to renal and hepatic tissue macromolecules. Pretreatment with inhibitors of the P-450 system decreased covalent binding and protected against histologic tissue damage. Acetaminophen was converted by rat kidney microsomes to reactive metabolites that became covalently bound to microsomal protein. The covalent binding required NADPH and was inhibited by carbon monoxide. These experiments suggest that acetaminophen overdoses in man occurs by a similar mechanism. Studies are now in progress to determine the significance of these observations to the pathogenesis of analgesic nephropathy.

PHS-6040
(12-75)

Project Description:

Objectives: Acetaminophen (p-hydroxyacetanilide) is a widely used mild analgesic and antipyretic that can result in life threatening hepatic necrosis and acute renal failure when ingested in large amounts. Acetaminophen and its glucuronide and sulfate conjugates are also the major metabolites in man of the analgesic, phenacetin (p-ethoxy acetanilide) which has been implicated in the development of the chronic renal lesion, analgesic nephropathy. Previous studies from this laboratory have demonstrated that acetaminophen can be metabolized by hepatic mixed function oxidases to a reactive electrophilic species capable of binding to tissue macromolecules resulting in hepatocellular necrosis.

We have therefore, investigated the possibility that extra-hepatic activation of acetaminophen by the kidney might occur in a similar manner resulting in renal tissue damage.

Methods Employed: Radiolabeled ^{3}H-(general) p-hydroxyacetanilide (acetaminophen) was obtained from New England Nuclear and purified by thin layer chromatography. Ring labeled ^{14}C-acetaminophen was obtained from New England Nuclear; ^{14}C-carbonyl acetaminophen was prepared from p-aminophenol and 1-^{14}C-acetyl chloride by a published procedure.

Male Fischer rats received subcutaneous injections of acetaminophen in water with pretreatments as follows: 1) phenobarbital (75 mg/kg, i.p. daily for 3 days), 2) cobaltous chloride (30 mg/kg s.c. twice daily for 2 days), or 3) diethyl maleate (450 mg/kg, 1:1 in sesame oil s.s., 60 min prior to injection). Animals were sacrificed at 24 hours with hepatic and renal tissues processed and scored for histologic damage according to previously described methods. In vivo metabolite binding studies were performed with Fischer rats, pretreated as above, receiving ^{3}H- (general) acetaminophen. Animals were sacrificed at 1 or 3 hrs. and the covalent binding of reactive intermediates determined according to previously published methods. In vitro binding studies were performed with microsomes and the radiolabeled compounds in the presence or absence of NADPH cofactor and other modifiers of the system. Covalent binding of reactive intermediates to microsomal protein was determined according to previously published methods.

Major Findings: Histologic renal damage consistently occurred in rats receiving large (750-1250 mg/kg), subcutaneous doses of acetaminophen. Pretreatment with cobaltous chloride protected against both renal and hepatic lesions while diethyl maleate appeared to potentiate damage in both organs. Covalent binding to both renal and hepatic macromolecules was observed following the in vivo administration of ^{3}H-acetaminophen. Decreased levels of binding to both tissues occurred in cobaltous chloride-pretreated animals. Diethyl maleate pretreatment resulted in enhanced hepatic binding at 1 and 3 hours with a slight increase in renal binding at 1 hr. but decreased binding at 3 hours. Incubations containing rat kidney microsomes, radiolabeled acetaminophen and an NADPH-generating system showed irreversible binding of radiolabel to microsomal protein. Omission of the NADPH-generating system decreased binding by 60-65%. Binding also appeared to be inhibited by carbon monoxide and by the exclusion of oxygen.

Identical amounts of covalent binding occurred with 1-^{14}C-carbonyl-, and ring-^{14}C-p-hydroxyacetanilide. Quantitatively similar binding per mg microsomal protein also occurred with microsomal preparations from Sprague-Dawley and Fischer rats although the renal microsomes from the latter strain contained 1.6 x more cytochrome P-450 per mg of microsomal protein.

Significance to Biomedical Research and the Program of the Institute: Acetaminophen is a widely employed minor analgesic and antipyretic. Our results demonstrate that acetaminophen can be converted by the kidney *in vivo* and by renal microsomes *in vitro* to arylating agents capable of covalently binding to renal tissue macromolecules. The metabolic pathways appear to involve cytochrome P-450 oxidation by enzymes located in the renal cortex.

It appears likely that the acute tubular necrosis observed in acetaminophen intoxication in man results from this mechanism but the possible contribution of acetaminophen activation in the pathogenesis of analgesic nephropathy is unclear at the present time.

Proposed Course of Project:

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00908-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Treatment of Essential Hypertension with Propranolol

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	D. Rollins	Research Associate	LCP NHLI
Other	W. Snodgrass	Guest Worker	LCP NHLI
	A.A. Taylor	Clin. Assoc.	HEB NHLI
	R. Rosen	Clin. Assoc.	HEB NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Snodgrass holds an NIH Research Fellowship in the National Inst. of Arthritis, Metabolism and Digestive Diseases.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Plasma concentration, plasma renin activity and antihypertensive effect of propranolol were measured in patients with essential hypertension. Plasma renin activity was maximally lowered at plasma concentrations of propranolol that did not lower blood pressure in most patients.

PHS-6040
(12-75)

Project Description:

Objectives: Propranolol, a beta adrenergic blocking drug, is useful in the treatment of hypertension. Because propranolol lowers plasma renin activity (PRA) it has been suggested that this mechanism is responsible for its antihypertensive action. The purpose of this study is to measure the blood pressure lowering effect, the PRA lowering effect and the plasma propranolol level in patients with essential hypertension. The objective was to learn if the lowering of the PRA was related to the antihypertensive effect of propranolol.

Methods Employed: Twenty-five patients with essential hypertension were studied on doses of propranolol from 80 mg to 480 mg daily. At each dose the PRA was measured by radioimmunoassay and the plasma propranolol level was measured fluorometrically. Blood pressure was measured in the outpatient clinic as well as by each patient at home.

Major Findings: As the dose of propranolol was increased there was a proportional rise in the plasma propranolol level. A dose of 80 mg daily (in four divided doses) was associated with a significant decrease in the PRA of all patients. In most patients on this dose the PRA was maximally decreased. However, only 8 patients on this dose showed a maximal antihypertensive effect. The general finding was that despite low PRA after a low dose of propranolol most patients required 160 mg to 320 mg of propranolol daily to control blood pressure. In addition it was found that despite a constantly low PRA the blood pressure began to rise after several weeks of propranolol therapy. These patients were often successfully treated by the addition of a diuretic to their therapy.

Significance to Biomedical Research and to the Program of the Institute: These studies demonstrate that the PRA-lowering effect of propranolol is not solely responsible for the antihypertensive effect of that drug. Most patients appear to maintain better blood pressure control when propranolol is combined with a diuretic.

Proposed Course of Project: Further studies are needed to determine if prolonged treatment with propranolol will lead to significant levels of its major metabolite, 4-hydroxypropranolol, which may contribute to its antihypertensive effect.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00910-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Development of Animal Model for Isoniazid Hepatitis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	D. Rollins	Res. Assoc.	LCP NHLI
Other:	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP, NHLI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

SUMMARY OF WORK (200 words or less - underline keywords)

An animal model for isoniazid hepatitis is being sought. Thus far guinea pigs treated with 25, 50, 75, 100 and 125 mg/kg for four days have failed to demonstrate hepatitis. Larger doses and longer treatment periods are being tried.

PHS-6040
(12-75)

Project Description:

Objectives: Previous studies in this laboratory have shown that acetylhydrazine, a metabolite of isoniazid, is hepatotoxic to the rat. However, liver toxicity could be demonstrated with isoniazid only after repeated hourly injections. The purpose of this project is to develop an animal model for isoniazid hepatotoxicity.

Methods Employed: Male NIH guinea pigs were administered isoniazid in doses of 25, 50, 75, 100 and 125 mg/kg ip for four days. Twenty-four hours after the last dose the livers were removed and tissue sections fixed and stained for microscopic examination.

Major Findings: Seven or eight animals were studied at each dose. At no dose was there any evidence of an inflammatory reaction of liver necrosis. At the 100 and 125 mg/kg doses minimal fatty changes were evident.

Significance to Biomedical Research and to the Program of the Institute: An animal model that resembles the hepatitis produced by isoniazid would be a useful aid in determining the mechanism involved. More importantly if an animal model were available, it should be possible to develop antidotes for preventing this lesion in patients taking isoniazid for the prevention and treatment of tuberculosis.

Proposed Course of Project: Further studies are needed to determine if larger doses and longer treatment periods with isoniazid will produce a hepatitis-like lesion in animals. Another animal species such as primates may be necessary to produce the isoniazid lesion.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00911-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Development of Specific Assays for Propranolol and 4-Hydroxypropranolol

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	D. Rollins	Research Assoc.	LCP NHLI
Other	J.R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI
	S.D. Nelson	Staff Fellow	LCP NHLI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Sensitive and specific assays for propranolol and its metabolite, 4-hydroxy-propranolol, have been developed using electron-capture gas chromatography and high pressure liquid chromatography. These methods will permit the rapid measurement of this drug in the plasma of patients in our outpatient hypertension clinic.

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PHS-6040
(12-75)

Project No. Z01 HL 00911-01 LCP

Project Description:

Objectives: Because of differences in its rate of metabolism there is a 20-fold interindividual variation in plasma propranolol concentrations. We routinely monitor plasma propranolol concentrations in our outpatient clinic with a fluorometric assay. This assay is nonspecific because of interference with propranolol metabolites (which are also effective antihypertensives). The assay also lacks sensitivity because of high plasma blank fluorescence. Thus, a rapid, sensitive and specific propranolol assay is needed to monitor the concentrations of propranolol and its metabolites in plasma.

Methods Employed: Gas chromatographic Assay: Plasma samples are extracted with benzene and propranolol is derivitized with trifluoroacetic anhydride. The derivative is then injected onto a 3% OV-17 column in a gas chromatography equipped with an electron capture detector.

High pressure liquid chromatographic (HPLC) assay: Plasma containing propranolol is extracted with benzene. The benzene is blown off with nitrogen gas and the residue dissolved in mobile phase to be used on the HPLC. The mobile phase is acetonitrile and water (1:1). A μ Bendapak C_{18} Waters column is used. A fluoromonitro detector increases the sensitivity and specificity of the assay for propranolol.

Major Findings: The gas chromatography-electron capture assay is quite sensitive with concentrations as low as 100 picograms measured. It is also very specific. The major metabolite of propranolol, 4-hydroxypropranolol, can also be measured with this method. The HPLC assay, while not as sensitive, is less time-consuming since a derivitization step is not required. With this method plasma levels of 1 ng/ml of propranolol can be measured. The metabolites of propranolol can also be measured with the HPLC assay.

Significance to Biomedical Research and to the Program of the Institute: These methods will enable us to rapidly and accurately measure the propranolol concentrations in our hypertensive patients. In addition, we can now compare efficacy of oral propranolol versus plasma concentrations of the drug and its vasoactive metabolites in all patients. Thus, we now can readily distinguish compliance problems in our patients. We also can determine which metabolites of propranolol are vasoactive.

Proposed Course of Project: Several patients that we have seen required much more propranolol than others to control their hypertension. The measurement of propranolol and its metabolites by the methods described in this project will be necessary to determine if these patients metabolize propranolol differently than those patients requiring smaller doses.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00912-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Liver and Kidney Necrosis Caused by Furosemide Administered Intravenously

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	D. Rollins	Res. Assoc.	LCP NHLI
Other:	A. Buckpitt	Guest Worker	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Buckpitt holds an NIH Research Fellowship in NHLI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Section on Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

SUMMARY OF WORK (200 words or less - underline keywords)

Furosemide administered intravenously to mice is a potent hepatic and renal toxin. There is a dose-threshold effect for intravenous furosemide but the threshold is lower than for intraperitoneal administration.

PHS-6040
(12-75)

Project No. Z01 HL 00912-01 LCP

Project Description:

Objectives: Furosemide has been found to be hepatotoxic in mice when administered intraperitoneally (ip). There appears to be a dose-threshold for the furosemide-induced hepatotoxicity similar to that observed for acetaminophen. However, the mechanism for this dose-threshold effect has not been determined. Glutathione is apparently not involved since doses of furosemide that are hepatotoxic do not deplete liver glutathione. These experiments were designed 1) to determine the extent of liver and kidney toxicity of furosemide administered intravenously (iv) and 2) to determine if furosemide administered iv exhibits a dose-threshold effect similar to furosemide administered ip.

Methods Employed: NIH male GP mice were used in all experiments. Furosemide was administered iv in doses of 50,100,200,250,300 and 400 mg/kg and ip in doses of 250,300 and 400 mg/kg to groups of 20 mice. Twenty-four hours later the livers and kidneys were removed, fixed and stained for microscopic examination.

Major Findings: Furosemide 400 mg/kg iv resulted in 100% mortality. Furosemide in a dose of 300 mg/kg iv resulted in 54% mortality but the remaining animals demonstrated extensive hepatic and renal necrosis. A dose of 300 mg/kg ip demonstrated only hepatic necrosis. There was a dose-threshold response for the hepatic and renal lesions when furosemide was administered iv but the threshold appears to occur at a lower dose than furosemide given ip.

Significance to Biomedical Research and to the Program of the Institute: Furosemide is a commonly used diuretic agent. It is important to determine the mechanisms responsible for the hepatic and particularly the renal lesions so that methods of prevention can be developed. The appearance of renal necrosis after large iv doses is especially important since doses up to 1,000 mg iv have been advocated for use in humans with acute renal failure, a recommendation that could produce even further renal injury in view of our results.

Proposed Course of Project: Further studies are required to determine if the hepatic and renal lesions induced by iv furosemide are the result of the formation of an active metabolite or a direct affect of furosemide. Such studies will include inhibition and enhancement of cytochrome P-450-dependent enzymes prior to furosemide treatment. The quantitative measurement of furosemide or a metabolite covalently bound to liver and kidney macromolecules after iv administration is also planned.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00913-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biliary Excretion of Acetaminophen and its Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	D. Rollins	Res. Assoc.	LCP NHLI
Other:	A. Buckpitt	Guest Worker	LCP NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Buckpitt holds an NIH Research Fellowship in NHLI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Section on Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

(GSH)

The active metabolite of acetaminophen is conjugated to glutathione/in the liver and excreted in the bile. Administration of ^{3}H-GSH before a toxic dose of acetaminophen results in the biliary excretion of radioactivity with a single chromatographic peak. These studies may lead to a better understanding of acetaminophen metabolism in animals and better methods of preventing acetaminophen-induced hepatic necrosis in man. More importantly, they may lead to a useful technique for evaluating the turnover, metabolism and synthesis of endogenous GSH in normal and diseased patients, a capability that is impossible at the present time.

PHS-6040
(12-75)

Project Description:

Objectives: Acetaminophen is a potent hepatotoxic drug in animals and man. Glutathione (GSH) protects liver cells against attack by the active metabolite of acetaminophen. Exogenously administered GSH will protect against the hepatic necrosis induced by acetaminophen. The purpose of this study is 1) to study the biliary excretion of acetaminophen and its metabolites in mice and 2) to determine if exogenously administered ^{3}H-GSH is conjugated with the toxic metabolite of acetaminophen.

Methods Employed: GSH ^{3}H-glycine (10 μCi) was administered intravenously to a mouse with a bile fistula. Bile was collected in 15 min fractions for 2 hr. In another mouse, acetaminophen (400 mg/kg ip) was administered 30 min after the ^{3}H-GSH and bile was again collected in 15 min fractions. Each 15 min bile collection was chromatographed on 500 μ DEAE cellulose plates. The plates were then divided into 0.5 cm sections and cellulose was scraped into a vial for scintillation counting.

Major Findings: When ^{3}H-GSH was administered alone no radioactivity was detected in the bile over a 2-hour period. However, when acetaminophen was administered intraperitoneally after iv ^{3}H-GSH, radioactivity appeared in the bile within 30 min. This radioactivity was associated with a single chromatographic peak.

Significance to Biomedical Research and to the Program of the Institute: A method for determining the in vivo acetaminophen metabolite conjugated to GSH will be helpful in studying acetaminophen metabolism. Similar methods applied to humans may be useful in determining ways of protecting against the hepatic necrosis seen in humans after large doses of acetaminophen. More importantly, such studies may provide a useful technique for assessing turnover, metabolism and synthesis of endogenous GSH in normal and diseased people, a capability which is not possible at the present time.

Proposed Course of Project: Further studies are planned to determine the effect of inhibitors and stimulators of drug metabolizing enzymes on the biliary excretion of acetaminophen. These studies may be applied to humans particularly if a stable isotope derivative of GSH can be made so that the mercapturic acid or the GSH conjugate of acetaminophen can be easily measured in the urine or bile.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00914-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of Chronic Propranolol Therapy on Anterior Pituitary Hormone Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	D. Rollins	Res. Assoc.	LCP NHLI
Other:	A.A. Taylor	Clin. Assoc.	HEB NHLI
	W. Snodgrass	Guest Worker	LCP NHLI
	R. Rosen	Clin. Assoc.	HEB NHLI
	J.R. Mitchell	Chief, Section on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Snodgrass holds an NIH Research Fellowship in the NHLI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Section on Clinical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

An interest in the central effects of propranolol has led to a study of the plasma levels of human growth hormone (HGH), prolactin (PL), follicle stimulating hormone (FSH), leutinizing hormone (LH) and thyroid stimulating hormone (TSH) in patients on chronic propranolol therapy for hypertension.

PHS-6040
(12-75)

Project Description:

Objectives: Propranolol, a beta adrenergic blocking drug, is clinically useful as an antihypertensive agent. It has been suggested that propranolol exerts some of its antihypertensive action through an as yet undefined central mechanism. This suggestion has been made because the beta blockade and plasma-renin-activity-lowering actions of propranolol occur at lower doses than those required for blood pressure control. In addition, many patients taking propranolol complain of lethary, mental fatigue, visual hallucinations and vivid dreams. To determine some of the central effects of propranolol the plasma levels of human growth hormone (HGH), prolactin (PL), follicle stimulating hormone (FSH), leutinizing hormone (LH) and thyroid stimulating hormone (TSH) were measured in patients on chronic propranolol therapy for hypertension.

Methods Employed: HGH, PL, FSH, LH AND TSH were measured by radioimmunoassay methods. In an initial study, plasma levels of these hormones were measured prior to beginning propranolol therapy. Patients were initially started on 80 mg of propranolol daily in four divided doses. The dose was then increased to 160 mg daily, 320 mg daily and 480 mg daily in four divided doses until the patient was normaltensive (BP 150/90 mm Hg or less). At each dose the propranolol level was measured using a fluorometric method. At each dose the level of the above hormones was measured in the plasma.

In a follow-up study the levels of HGH, PL, FSH, LH and TSH were measured over a 3-hour period once when the patient was taking an antihypertensive dose of propranolol and again during a control period. The control period was either before propranolol therapy had been initiated or after the patient had been withdrawn from propranolol therapy. In all cases the plasma level of propranolol was measured at the beginning and end of the 3-hour period. HGH, PL and TSH were measured at the beginning and end of the 3-hour period. However, because of the pulsatile nature of their secretion, FSH and LH were measured every 20 min throughout the 3-hour period.

Major Findings: The initial study demonstrated definite increases in HGH levels in most patients on propranolol, but the indivudal variations in the other hormones made analysis difficult. Thus, the protocol of measuring the hormone levels over a 3-hour period of time was instituted. At this time only a few patients have been studied and their plasma hormone levels have not yet been assayed.

Significance to Biomedical Research and to the Program of the Institute: It is hoped that these studies will help to define some of the long-term effects of propranolol in hypertensive patients.

Proposed Course of the Project: Six patients on chronic propranolol therapy are to be studied in which the hormones are measured over a 3-hour period of time. These data will then be analyzed before further studies are planned.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00915-01 LCP

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Importance of Glutathione Transferases in the Toxicity of Reactive Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	A.R. Buckpitt	Guest Worker	LCP NHLI
	D.E. Rollins	Research Assoc.	LCP NHLI
Other:	J.R. Mitchell	Chief, Sec. on Clinical Pharm.	LCP NHLI

COOPERATING UNITS (if any)

Dr. Buckpitt holds an NIH Research Fellowship.
Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Clinical Pharmacology

INSTITUTE AND LOCATION

NIH, NHLI, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine how important GSH transferases are in the conjugation of reactive metabolic intermediates in vivo. We are currently engaged in a search for a compound with the following requirements: 1) The compound must be an effective GSH transferase inhibitor in vivo. 2) It must not deplete GSH from the liver. 3) It must prevent the depletion of GSH caused by hepatotoxins such as acetaminophen. 4) It must not inhibit the cytochrome P-450 drug metabolizing enzymes of the liver. If a compound meeting these requirements can be found, studies to determine whether the compound potentiates the liver toxicity of acetaminophen will be initiated.

PHS-6040
(12-75)

Project Description:

Objectives: Several recent reports from this laboratory have described the severe hepatic necrosis caused by high doses of acetaminophen. The extent of liver damage can be directly correlated with a decrease in the glutathione (GSH) content of the liver and an increase in the covalent binding of drug to tissue macromolecules. Preliminary studies have shown that cephaloridine, in sufficient doses, can prevent the depletion of GSH caused by high doses of pHAA. These results suggested that cephaloridine might be inhibiting the GSH transferase. If this were correct, then cephaloridine might be used as a tool to elucidate the role of GSH transferase in the conjugation of reactive metabolic intermediates. This research project was designed to determine whether the conjugation of reactive intermediates of hepatotoxic drugs is spontaneous or whether this process requires catalysis by GSH transferase.

Methods Employed: Cephaloridine (2,000 mg/kg, ip), indocyanin green (10 to 500 mg/kg, iv) and benziodarone (5 to 100 mg/kg, ip) were administered to groups of mice 5 min before the ip injection of 300 or 400 mg/kg acetaminophen. Two hours after the initial injection the animals were sacrificed and GSH levels were determined spectrophotometrically. Since cephaloridine was effective in reducing the depletion of GSH caused by acetaminophen, the amount of pHAA bound to tissue protein was determined using mice pretreated with cephaloridine.

Major Findings: The depletion of liver GSH caused by pHAA was significantly reduced in mice pretreated with cephaloridine. Subsequent studies on the covalent binding of acetaminophen showed that cephaloridine reduces the amount of labeled pHAA bound to liver protein. This suggests that, rather than acting as a GSH transferase inhibitor, cephalorine may be reducing the production of the reactive intermediate of pHAA by inhibiting the cytochrome P-450 system.

Indocyanin green (10 to 500 mg/kg iv) and benziodarone (5 to 100 mg/kg, ip), which have been shown to act as inhibitors of several of the GSH-S-transferases in vitro, failed to reduce the depletion of liver GSH in mice injected with pHAA.

Significance to Biomedical Research and to the Program of the Institute: This project will yield useful information on whether the metabolic intermediates of some of the hepatotoxins are capable of spontaneous conjugation with GSH or whether transferase enzymes are required for this process.

Proposed Course of Project: The search for a compound fulfilling the requirements described in the Summary is continuing.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00916-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Development of specific g.c.-m.s. assays for 16β-OH-DHEA and 18-OH-DOC.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.B. Franklin	Visiting Fellow	NHLI LCP
OTHER:	J. Mitchell	Chief, Section on Clinical Pharm.	NHLI LCP
	A.A. Taylor	Clinical Assn.	NHLI HEB
	S.D. Nelson	Staff Fellow	NHLI LCP

COOPERATING UNITS (if any)

Dr. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

A method has been described for the synthesis of tritiated 16β-OH-DHEA. The radioactive steroid has been administered to normotensive and low-renin hypertensive patients and an assay procedure developed to measure the excretion rate of this steroid in the urine of these patients before and after the administration of aminoglutethimide.

Studies have also been carried out on tritiated 18-OH-DOC after intra-venous dosing of normotensive and low-renin hypertensive patients. Attempts have been made to isolate and synthesize the proposed major urinary metabolite of 18-OH-DOC, namely 18-OH-TH-DOC using both in vitro and chemical techniques.

PHS-6040
(12-75)

Project Description:

Objectives: The phenomenon of low-renin essential hypertension has been equated with an excess of some unidentified mineralocorticoid steroid since, in most patients, measurements of aldosterone and other recognized mineralocorticoids such as 11-deoxycorticosterone, corticosterone, and 4-pregnen-18, 21-diol-3,20-dione (18-hydroxy-deoxycorticosterone; 18-OH-DOC) have failed to show consistently elevated values. However, the blood pressure of patients exhibiting the syndrome of low-renin hypertension may be normalized by treatment with aminoglutethimide, an inhibitor of steroid biosynthesis, and also by spironolactone, an antagonist of mineralocorticoid action. Preliminary evidence has indicated that the steroids androst-5-ene 3β,16β-diol (16β-hydroxy-dehydroepiandrosterone; 16β-OH-DHEA) and 18-OH-DOC might be responsible for the elevation of blood pressure in patients suffering from low-renin hypertension. Therefore, it was decided to develop assays to measure the secretory and excretory rates of these steroids in low-renin hypertensive patients before and after administration of aminoglutethimide.

Methods Employed: (I) [^{3}H]-16β-OH-DHEA. (a) Synthesis: The radioactive starting material, [^{3}H]-DHEA, was converted to androst-5-ene-3β-acetoxy-17-one, 17-enol-acetate utilizing isopropenyl acetate. The enol-acetate was converted to androst-5-ene-3β,16β-diacetoxy-17-one which was hydrolyzed both enzymatically and chemically to yield androst-5-ene-3β,16β-diol-17-one. The purity of the final product and the intermediates was examined by nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (m.s.) using both electron impact (e.i.) and chemical ionization (c.i.) techniques. Aliquots of final product were transferred to multiple-dosing vials using absolute ethanol and filtered through Millipore filters in a sterile atmosphere. The absolute ethanol was evaporated under a slow stream of dry N_2 also passed through a Millipore filter. The vials were submitted for pyrogen and sterility testing before administration of the radioactive compound to patients and normotensive controls.

(b) Assay: An aliquot of urine (20-25% of the total volume collected during the first 24 h. period after administration of radioactive material) was run over an Amberlite resin before being subjected to solvolysis and enzyme hydrolysis. The extract was further purified by thin-layer chromatography and derivatization with methoxime and silylation before being quantitated by gas chromatography (g.c.). The extracts were also examined by e.i. and c.i. mass spectroscopy and monitoring by a Multiple Ion Detector (m.i.d.) unit.

(II) [^{3}H]-18-OH-DOC. (a) This compound was purchased as such but subjected to extensive analysis and sterility testing before being administered to patients.

(b) Assay: Approximately 20% of the first 24 h. urine (collected after administration of the radioactive steroid) was passed over an Amberlite resin. The eluate from the column was treated with periodic acid and purified extensively by thin-layer chromatography before being examined by g.c. and g.c.-m.s. (both e.i. and c.i.-m.s.).

(c) Synthesis of the anticipated major metabolite of 18-OH-DOC in human urine, that is 5(α,β)-pregnan-3,18,21-triol-20-one (18-OH-TH-DOC) was attempted both

chemically, necessitating hydrogenation and reduction, and using an in vitro liver system prepared from the 10,000 xg supernatent of rat liver and also a rat liver acetone powder preparation. In all attempts, the starting material was 18-OH-DOC to which was added a tracer amount of tritiated 18-OH-DOC. The products isolated were examined by thin layer chromatography using radiochromatogram scanning to locate radioactivity, and by c.i. mass spectroscopy. Gas chromatography of the silyl derivatives of both 18-OH-DOC and 18-OH-TH-DOC was carried out on several stationary phases.

Administration of radioactive steroids [^{3}H]-16β-OH-DHEA and [^{3}H]-18-OH-DOC to patients: The dry sterile steroid previously distributed into a dose-vial and containing a known amount of radioactivity was reconstituted with absolute ethanol and diluted with 0.9% w/v sodium chloride solution to a known specific activity. The radioactive compound was administered intravenously via a heparin lock. The two radioactive compounds were given one after the other allowing flushing of the heparin lock with sterile normal saline. When appropriate, the steroids were given on the fourth day of aminoglutethimide treatment (3 weeks duration: 250 mg. q.6.h. p.o. Eliptin).

Excretion data: Urine was collected for 3 days in 24 hr. aliquots. Excretion of radioactivity for each patient was tabulated. Levels of sodium, potassium, 17-keto-steroids, creatinine, 17-hydroxy-steroids and aldosterone in urine were monitored as were the levels of sodium, potassium, renin, creatinine, aldosterone, cortisol, progesterone, 17-hydroxy-progesterone, 11-deoxycortisol and deoxycorticosterone in plasma. All urinary samples were kept at -20° C prior to analysis.

Major Findings: (I) 16β-OH-DHEA. Thus far, only excretion rates of 16β-OH-DHEA in 4 patients before and after administration of aminogluthemide have been studied. These have indicated that aminoglutethimide has an equivocal action on the excretion of this steroid. The major conclusion was that aminoglutethimide lowered the blood pressure in those patients suffering from low-renin hypertension without causing a reproducible decrease in 16β-OH-DHEA excretion in the urine. Thus the higher urinary level of this steroid (compared to normotensive controls) was not responsible for the hypertensive state of these persons.

(II) 18-OH-DOC. Progress in the assay for 18-OH-TH-DOC in the urine of low-renin hypertensive patients has been severely hampered by the lack of an authentic standard. Other groups of researchers have claimed to have isolated and quantitated this compound in the urine of low-renin hypertensives but have not presented any structural proof as to the identity of the compound they have been measuring. Several attempts have been made in this laboratory to copy exactly the isolation procedures used by these other workers, except that intermediate products in the isolation were subjected to both e.i. and c.i. mass spectrometry. It was found that (a) the methodology was not reproducible and (b) the final compound did not appear to be 18-OH-TH-DOC. One synthetic procedure produced a compound that gave a mass spectrum (c.i.) which appeared to show the loss of the loss of the elements of water from 18-OH-TH-DOC but the exact structure of the compound could not be determined. Several other syntheses have produced unidentifiable material and attempts to synthesize

the 18-OH-TH-DOC by methods reported in the literature have not been successful.

Significance to Biomedical Research and the Progress of the Institute: The controversy as to whether low-renin hypertension is caused by a mineralocorticoid excess or by other physiological factors has not yet been resolved. The development of specific methods for 16β-OH-DHEA and 18-OH-DOC as described in this report should elucidate the role, if any, of these mineralocorticoids in the pathogenesis of low-renin essential hypertension.

Proposed Course of Project: The role of the steroids 16β-OH-DHEA and 18-OH-DOC in the syndrome of low-renin hypertension will continue to be studied using the parameters previously mentioned and the assay procedures alluded to in this report.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00917-01 LCP

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Studies on the Formation of Reactive Metabolites of Phenacetin and Acetaminophen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jack A. Hinson	Staff Fellow	LCP NHLI
OTHER:	Sidney D. Nelson	Staff Fellow	LCP NHLI
	Jerry R. Mitchell	Chief, Section on Clinical Pharmacology	LCP NHLI

COOPERATING UNITS (if any)

Dr. Hinson was supported by NIH Research Fellowship 1 F 22 HL 2206-01.
Dr. Nelson is funded by the PRAT Program of the NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Clinical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of nephrotoxicity and hepatotoxicity of phenacetin are unknown. When phenacetin is incubated with microsomes a reactive arylating metabolite is produced. Since the hepatotoxicity of acetaminophen has been shown to be mediated through the formation of a reactive metabolite, the production of this metabolite of phenacetin was investigated and compared to the formation of the reactive metabolite of acetaminophen. Both metabolites are produced by a cytochrome P-450 mixed function oxidase. The production of the phenacetin metabolite is stimulated by pretreatment of animals with phenobarbital, whereas, the production of the acetaminophen metabolite is stimulated by pretreatment with 3-methylcholanthrene. When the arylating metabolites of acetaminophen and phenacetin were trapped by including the alternate nucleophile glutathione in the microsomal incubations and both conjugates isolated, they were identical: phenacetin had become de-ethylated. When both glutathione conjugates were trapped in the presence of an oxygen-18 atmosphere and examined by mass spectrometry, oxygen-18 was found to be incorporated into the conjugate of phenacetin, but not into the conjugate of acetaminophen. Thus the arylating metabolite of acetaminophen is formed by N-hydroxylation, whereas the arylating metabolite of phenacetin is formed by epoxidation.

PHS-6040
(12-75)

Project Description:

Objectives: The mechanism of phenacetin-induced nephrotoxicity and hepatoxicity are unknown. Previous studies in this laboratory have shown that the acetaminophen-induced hepatoxicity results from the formation of a reactive arylating metabolite produced by oxidation of the parent drug by a cytochrome P-450 mixed-function oxidase. When ^{3}H-phenacetin is incubated with microsomes a reactive arylating metabolite is also produced. We therefore examined the mechanism of production of this metabolite and compared it to the production of the arylating metabolite of acetaminophen.

Methods Employed: Microsomes were isolated from hamsters by standard procedures. The alternate nucleophile, glutathione, was used to trap the reactive species in large microsomal incubations. Mass spectrometry was used to determine the structure of the glutathione conjugates. All other methods were standard procedures.

Major Findings: A comparison of the formation of the reactive metabolite of phenacetin to the formation of the reactive metabolite of acetaminophen showed they arose by different mechanisms. Pretreatment of hamsters with the polycyclic hydrocarbon 3-methylcholanthrene increased the *in vitro* covalent binding of acetaminophen and decreased the *in vitro* covalent binding of phenacetin. Where as pretreatment of hamsters with phenobarbital increased the *in vitro* covalent binding of phenacetin but did not alter that of acetaminophen. The formation of the reactive metabolite of phenacetin was dependent on NADPH and molecular oxygen and was inhibited by carbon monoxide indicating that it was catalyzed by a cytochrome P-450 mixed-function oxidase.

Trapping experiments were run by including the alternate nucleophile, glutathione, in the microsomal incubations of phenacetin and acetaminophen. Under these conditions the covalent binding to protein of phenacetin and acetaminophen decreased and a glutathione conjugate was formed. Isolation of a conjugate from the incubation of phenacetin and from the incubation of acetaminophen showed both conjugates to be identical: phenacetin had become de-ethylated. When the glutathione conjugate of phenacetin was trapped in the presence of an oxygen-18 atmosphere and the metabolite examined by mass spectrometry, oxygen-18 was found to be incorporated into the conjugate. No oxygen-18 was incorporated into the glutathione conjugate of acetaminophen.

Significance to Biomedical Research and the Program of the Institute: The present studies demonstrate that phenacetin is activated *in vitro* to a reactive metabolite possibly an epoxide, that differs from the N-hydroxy metabolite formed from acetaminophen. Since the toxicity of phenacetin may proceed through reactive metabolites, the relative amounts of reactive metabolite arising through possible epoxidation of phenacetin will be determined.

Proposed Course of Project: The *in vivo* importance of the epoxidation of phenacetin in the production of a reactive arylating metabolite will be investigated.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
NATIONAL HEART AND LUNG INSTITUTE

July 1, 1975 through June 30, 1976

The Laboratory is heavily involved in the development and application of newer methods of structural analysis to the elucidation of structures of compounds of biological interest brought to us from within the Heart and Lung Institute and NIH in general. It is still the case that the available techniques, nmr, mass spectrometry, and X-ray crystallography, require more material in a relatively purer form than is often available in the course of biological experimentation within NIH, so that we do have sufficient resources to also investigate suitable biochemically oriented problems brought to us from the outside. We have always chosen this approach since thereby our facilities and skills are continually in a stage of improvement through use and our intramural value should increase proportionally. The techniques of mass spectrometry and nmr are cases in point: both are now considered almost routine instruments in studies within NIH, even though at first they were applied almost exclusively to "outside" chemically oriented problems.

Our research in these areas and in X-ray continues. This year we have finally begun testing a field desorption source built in our shop. There are many technical difficulties, however, the sensitivity is low and mass identification is difficult. A mass marker has been constructed to help in the latter problem and is working satisfactorily.

Analysis of mass spectral data is tedious and we have been further involved this year in the Chemical Information System (DCRT) of computerized bibliographic and file searching to speed up and simplify identification of unknown compounds. Today we are in a very advantageous situation in this respect and problems involving even complex mixtures of simple unknowns amenable to GC-MS often are solved in a few hours. However, we must still translate the information from the spectrometers to the computers by hand and, although this has certain advantages in difficult cases, it represents a waste of time and facilities in others. We are currently working to interface the spectrometers directly through our laboratory computer (PDP-8) to the DCRT PDP-10 to overcome this obstacle.

We have recently received delivery on a JEOLCO nmr spectrometer (^{13}C and ^{1}H) that should considerably lighten the load and improve use of our XL-100 spectrometer since it has features simplifying operation. We intend that it be operated by as many individual investigators as possible at NIH, in an effort to increase use of this valuable and relatively straightforward technique.

In the area of X-ray crystallography, activity has centered around improvements in methods of calculation such as the quartet invariant techniques that allows analysis of even larger and more complex structures. Forming a crystal successfully is still a major difficulty in applying this technique to biological problems, however, during this year Dr. Silverton and his

group have been able to complete more than 6 complete structures using these newer techniques.

Activities of the recently added Section on Physiological Chemistry included studies on the kallikrein-kinin system, prostaglandins, mediators of the allergic response in lung and methods for the microanalysis of peptides and proteins.

Kallikrein-Kinin System: Human high molecular weight kininogen is required for surface activation of Hageman factor (factor XII). Our highly purified B4β and B4γ kininogens when used at 10% of their concentration in normal plasma completely corrected the clotting and other defects in Williams plasma, which is congenitally deficient in both biological and immunological kininogen activities. The B4 kininogens also enhance 2- to 3-fold the ability of active Hageman factor to activate its three natural substrates, PTA, plasminogen pro-activator, and prekallikrein. In addition to kininogen and kallikrein, blood may contain another protein required for the optimal rate of Hageman factor activation. Pepsitocin, the kinin released from human kininogens by pepsin, has been purified to homogeneity by IRC-50 adsorption, SP-Sephadex C-25 chromatography, and Bio-Gel P-4 gel filtration. Quantitative amino acid analysis gave the theoretical values for Met-Lys-bradykinin (MLBK). It is highly probable that MLBK found in urine acidified after collection is formed by uropepsin as pepstatin blocks the formation of MLBK. Men excrete more uropepsinogen and MLBK than women. Total kinins were <0.1% normal in the urine of a subject with no detectable plasma kininogen and <1% normal in another subject who had 9% kininogen. Kininogen has been detected in the urine of normal subjects. The amount of kinin contained in "urokininogen" is from 50-90% the total kinins in urine. Kallikrein has been localized in the distal tubular cells of rat kidney cortex by means of fluorescent mono-specific antibody to rat urinary kallikrein.

Prostaglandins (PG's): We previously reported that PGA_1 is converted by human red blood cells to two GSH conjugates ($GSH \cdot PGA_1$ and reduced conjugate). Red blood cell suspensions, lysed red blood cells, and purified preparations of 9-hydroxyprostaglandin dehydrogenase convert $GSH \cdot PGA_1$ to the reduced form. With the purified enzyme, reduction of $GSH \cdot PGA_1$ is 24,000 times more rapid than that of PGE_1, the presumed natural substrate. PGA_1 infused into the canine renal artery appears in urine as a mixture of polar forms tentatively identified as the cysteine and N-acetylcysteine conjugates of PGA_1. PGE_1 and $F_1\alpha$ are not converted to polar metabolites in similar experiments. Women's urine contains a conjugated form of PGA_2 which may be related to $GSH \cdot PGA$. Culture fluid of rabbit renal interstitial cells contains dihomo PGE_2, a previously unreported C_{22} prostaglandin.

Mediators of Allergic Response in Human Lung: Studies have continued on the identification of mediators released from in vitro human lung preparations by antigen-antibody reactions. Addition of arachidonic acid, a substrate for prostaglandin synthetase, to tissue supernatants inhibited the release of both histamine and slow reacting substance of anapylaxis (SRS-A). These data provide an explanation for the well-known aspirin sensitivity seen in about 10% of adult asthmatics since in these patients elevated prostaglandin production may be inhibiting mediator release. Usually, challenged human

lung releases mainly prostaglandin E's, which relax human bronchi. Previously, the SRS-A in the lung tissue supernatants was separated into four biologically active fractions. A simple method has been devised for the conversion of Fractions II, III and IV into Fraction I which is chloroform soluble. The SRS-A's, like thiols and disulfides, are sensitive to light and susceptible to oxidation but SRS-A contains neither a thiol nor a disulfide. The marked lability of SRS-A to reduction with sodium borohydride or Raney nickel suggests the presence of an aldehyde, ketone or sulfoxide grouping. Studies on the biosynthesis of SRS-A in monkey lung indicate that both sulfur-containing compounds, such as cysteine, sodium sulfide and methionine, and carboxylic acids, such as succinic and α-keto-glutaric, are capable of enhancing SRS-A production. However, these two classes of compounds may increase SRS-A activity by different mechanisms, the sulfur-containing compounds acting upon membranes to affect release while the carboxylic acids could be incorporated directly into the SRS-A molecule.

Peptides: A new dipeptidyl aminopeptidase has been discovered which cleaves amide bonds involving the imino group of proline. When used in conjunction with the previously reported DAP-I and IV, it should be possible for the first time to confidently degrade any polypeptide. The use of the combined DAP enzymes and our GC-MS method for dipeptide analysis appears to be a superior sequencing technique whose major assets are sensitivity and speed. The high performance liquid chromatographic technique for amino acid PTH analysis has been refined to the point where it is now possible to resolve all 20 derivatives in under 15 min. Porcine villikinin, like canine villikinin is a 2,000 m.w. polypeptide. It has been partially purified using Dowex 50 and BioGel P4 columns and droplet countercurrent chromatography. New methods based on fluorescamine have been developed for picomole detection of amino acid, peptides and related compounds on TLC plates. Newly developed (elsewhere) TLC plates containing a special blend of fluorophores were evaluated and found especially useful in the TLC analysis of UV-absorbing amino acid derivatives.

360a

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01002-04 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Nuclear Magnetic Resonance of Natural Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: E. A. Sokoloski Chemist CH NHLI

OTHER:

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this work has been to apply Nuclear Magnetic Resonance spectroscopy for structural studies. Carbon-12 and phosphorus-31 resonance phenomena have been used to study such systems as a) Phospholipids, b) antineoplastic agents like Daunomycin, c) enzymes carboxy peptidase. In each case, information about chemical structure or the mode of interaction of these biochemical systems should lead to a description of specific interaction in natural systems.

PHS-6040
(12-75)

Project No. Z01 HL 01002-04 LC

Project Description:

Phospholipids have been studied by ^{31}P spin-lattice relaxation (T_1) measurements, the object of these studies being to compare the T_1 of lipid vesicles with those of intact native lipoproteins. Since relaxation times are related to molecular motions of a particle, correlation between secondary structural interaction and this NMR data should prove useful as a structural probe. Paramagnetic probes were also introduced into lipid and lipoprotein solutions, demonstrating that both native HDL and LDL are micelles rather than bilayers.

A collaborative study with Dr. C. Storm and P. Evans of Howard University has focused on the interaction of carboxy peptidase with a carbon-13 enriched peptide glycyl-tyrosine. Expectations are that the carbon-13 NMR spectra of the peptide will provide information on the mode of interaction of the metal activated peptidase.

In early stages are also carbon-13 studies of adriamycin and daumomycin metal binding. By examining the effects of paramagnetic probes on the carbon skeleton, we hope to obtain information about the conformation of these experimental antineoplastic agens in solution. This study is being undertaken as a collaborative effort with Dr. C. Chignell of NHLI.

Publications:

Hiranaka, P. K., Kleinman, L. M., Sokoloski, E. A., and Fales, H. M. Chemical structure and purity of dyes used in lymphograms. Amer. J. Hosp. Pharm 32: 928, 1975.

Irreverre, F., Wilson, E., Fales, H, M., Sun, T., and Sokoloski, E. A. Isolation of β(2 furayl) ℓ-alanine from hydrolyzed extracts of Koetreuteria pariculata (golden rain tree) seeds. Lloydia 38: 178-180, 1975.

Highet, R. J., and Sokoloski, E. A. Structural investigations of natural products by newer methods of NMR spectroscopy. Fortsch der Chem organ Natur 32: 119-166, 1975.

Kapadia, G. J., Paul, B. D., Silverton, J. V., Fales, H. M. Sokoloski, E. A. Melochinone, a novel quinolinone from Melochia Tomentosa L J. Amer. Chem Soc. 97: 6814-6819, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01003-05 LC

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure of National Products using Instrumental Methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. M. Fales	Chief, Lab. of Chem.	CH NHLI
OTHER:	T. Jaouni	Chemist	CH NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

SUMMARY OF WORK (200 words or less - underline keywords)

The structures of a wide variety of products found in insect secretions and carcinogenic plant products have been elucidated and in some cases synthesized using gas chromtography-mass spectrometry.

Confusion in analysis of overdose samples due to aspirin has been studied and overcome. A series of high molecular weight standards for mass spectrometry have been synthesized and a new technique to detect mixtures in chemical ionization mass spectrometry has been developed.

PHS-6040
(12-75)

Work continues on the isolation and characterization of the peptide responsible for allergenic activity in fire ant venom with H. Baer (Bur. Biol. Std.). An initial amino acid analysis indicates that only one peptide may be present but this surprising finding requires confirmation.

With M. Blum (Univ. of Georgia) an interesting series of compounds has been discovered in defense and sex glands of millipedes and true bugs. These include the unusual compound benzoyl cyanide, acetosyringone, o-cresol, benzyl alcohol, syringaldehyde, and vanillin. A new pheremone (4,6-dimethyl-3-nonanone) in the velvet ant vespula maculatum has been discovered and successfully synthesized: and separated into its threoid and erythroid isomers.

With G. Kapadia the tumorogenic plant melochia tomentosa has been investigated further and a new series of unusual isatin alkaloids discovered. The structure of the first one is nearing completion using now-classical methods of NMR and mass spectrometry. The compounds have cytotoxic activity in preliminary tests.

In continuing investigations of problems involved in overdose analysis, we (Agnes van Langenhove, guest worker, Ghent, Belgium) have investigated the complexities and anomalies due to excessive amounts of aspirin in the samples. This has disclosed the fact that aspirin acetylates amines easily in the gas chromatograph. Furthermore, a general gas chromatographic anomaly has been disclosed, wherein compounds of higher molecular weight may appear in the GC-MS at elution times far earlier than expected. This is due to reaction at the end of the GC column and may occasionally be the source of considerable confusion in GC-MS analysis.

A series of very high molecular weight ($\sim$4,000) standards have been synthesized and analyzed for use in mass spectrometry (via field desorption and chemical ionization). They may also be useful in gel permeation chromatography, etc., since they have precisely known molecular weights.

An unusual effect has been noted in chemical ionization mass spectrometry when larger amounts of sample are used, involving the formation of protonated dimers. The amount of dimer formed is structurally dependent and serves as a means of distinguishing between spectrum complexity due to fragmentation as opposed to the presence of more than one component in mixtures without the use of gas chromatography (Graeme Wright, U. of New Zealand).

Publication:

1. Heller, S. R., Koniver, D. A., Fales, H. M. and Milne, G. W. A.: A conversational mass spectra system III. Display and plotting of spectra and dissimilarity comparison. Anal. Chem. 46:947-950, 1974.

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3. Das, K. G., Fales, H. M. and Beckey, H. D. Electron ionization, chemical ionization and field desorption spectra and pyrolysis of isomeric 3,5,6-triphenyl 4-nitro-Δ^1-cyclohexenes. Int. J. Mass Spec. 15:110-112, 1974.

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7. Grantham, P. H., Mohan, L. C. Weisburger, E. K., Fales, H. M. Sokoloski, E. and Weisburger, I. H. Identification of new water-soluble metabolites of acetanilide. Xenobiotica 4:69-76, 1974.

8. Kapadia, G. J., Paul, D. B., Silverton, J. V., Fales, H. M. and Sokoloski, E. A. Melochinone, a novel quinolinone from Melochia tomentosa L. J. Amer. Chem. Soc. 97:6814-6819, 1975.

9. Gross, M., Baer, H., and Fales, H. M. Urushiols of poisonous anacardiaceae. Phytochemistry 14:2263-2266, 1975.

10. Irreverre, F., Wilson, E., Fales, H. M., Sun, T., Sokoloski, E. Isolation of β-(2-furoyl)-1-alanine from the hydrolyzed extracts of Koelreuteria paniculata (golden rain tree) seeds. Lloydia 38: 178-180, 1975.

11. Pant, R. and Fales, H. M. Occurrence of a new amino acid in Crotalia seeds. Phytochemistry 13:1626-1627, 1974.

12. Hiranaka, P. K., Kleinman, L. M., Sokoloski, E. A., and Fales, H. M. Chemical structure and purity of dyes used in lymphograms. Amer. J. Hosp. Pharm. 32:928, 1975

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01004-05 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization of natural products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. A. Lloyd Research Chemist CH NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the structures of new compounds of natural origin which may have physiological properties. Plant constituents of Acacia vilos, Diospyros virginiana, Krameria ixica, Sassafras albidum, melochia tomentosa (potential carcinogens) and Schinus terebinthifolius (respiratory and allergic reactions) were determined. The pheromones of various species of ants and termites were studied.

PHS-6040
(12-75)

Project Description;

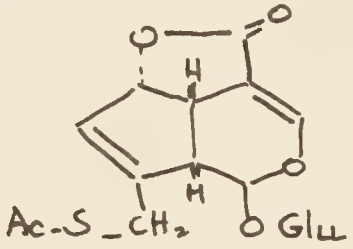
1. Insect Pheromones (in collaboration with M. S. Blum, University of Georgia)

The glandular extracts of some ants and termites were investigated with the aid of high pressure liquid chromatography and combined gas chromatography-mass spectrometry. Some of the identified new products were synthesized for further examination.

The pheromones secreted by over twenty species of Australian primitive ants were determined and several new esters were synthesized (in collaboration with C. P. Haskins, Carnegie Institution) A number of rare Myrmecocystus ants were also investigated.

2. Plant products

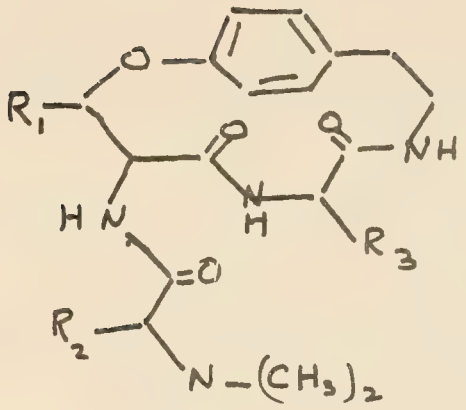
Suspected carcinogenic plant substances were studied (in collaboration with G. J. Kapadia, Howard University). The investigation of Acacia villosa, Diospyros virginiana and Krameria ixina was continued. With the aid of mass spectrometry the structures of some iridoid glucosides of Paederia fetida were established. A sulfur containing iridoside was among the products isolated.

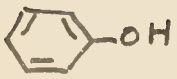


In addition, long chain hydrocarbons and alcohols, steroidal acids and alcohols and methyl mercaptan were identified. A large number of potential carcinogens were identified in Sassafras albidum.

The study of the volatiles of Schinus terebinthifolius suspected of being responsible for respiratory troubles and allergic reactions in Florida was continued. In addition to the eleven volatile monoterpene hydrocarbons previously characterized, the berries were shown to contain over fifty sesquiterpene hydrocarbons, alcohol and ketones and several triterpenes. A number of these were identified.

In an investigation of the alkaloids of Melochia tomentosa, several peptide alkaloids were isolated (with G. J. Kapadia, Howard University). The structure of three of these alkaloids was determined through degradation by acid and alkaline hydrolysis together with amino acid analysis and mass spectrometry. I and II are new compounds and III (scutianin B) has been reported previously in another plant.



I $R_1=R_2=CH(CH_3)_2$ $R_3= -CH_2$ 

II $R_1=R_2= -CH(CH_3)_2$ $R_3= -CH_2CH-(CH_3)_2$

III $R_1=-CH(CH_3)_2$ $R_2=R_3= -CH_2$

Publications:

Lloyd, H. A., Blum, M. S. and Duffield, R. M. Chemistry of the male mandibular gland secretion of the ant Camponotus clarithorx. Inst. Biochem 5: 489, 1975.

Silverton, J. V., and Lloyd, H. A. The crystal and molecular structure of the non-morphinoid narcotic analgesic, Viminol. Acta Cryst. B31: 1576, 1975.

Bright, W. M., Lloyd, H. A., and Silverton, J. V. The crystal structure and absolute configuration of astrocasine methobromide. J. Org. Chem. in press.

Kapadia, G. J., and Lloyd, H. A. Constituents of Sassafras albidum. Phytochem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01005-05 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

X-ray Structural R&D for Physiologically Important Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. V. Silverton	Research Chemist	CH NHLI
OTHER:	O. W. Adams	Guest Worker	NSF
	T. Lee	Student	MIT
	C. Kabuto	Guest Worker	CH NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	2.0	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

In addition to crystallographic studies where the X-ray method has either been the quickest or only conclusive technique for settling chemical problems, considerable further work has been done in the application and development of quartet invariant techniques mentioned in the previous report.

370

PHS-6040
(12-75)

PURPOSE:

Investigations by X-ray crystallography of molecules of interest chemically or physiologically with emphasis on problems where other methods are inconclusive. Development of techniques in direct methods.

A) The structure and absolute configuration of chetoglobosin

This toxic mold product has a molecular weight of 516. This project proved resistant to all previously reported direct methods techniques. One might go so far as to challenge anyone to solve the problem by any automatic or semi-automatic method. A novel application of quartet invariants was successful. The structure has been refined to an R-factor of 0.032 and, despite the low oxygen content, the absolute configuration has been determined with an apparent probability of 0.999.

B) The structure of viridicatum toxin (with C. Kabuto).

This project was initiated with only the molecular formula, molecular weight (598) and the fact that the compound absorbed in the UV as basic information. Methods as in a) were successful for this structure which also resisted solution by standard methods. The compound proves to be a novel derivative of tetracycline and has been refined to an R-factor of 0.050. The absolute configuration has not been determined as yet but the measurements will be done shortly and it is expected that, since there are 10 oxygen atoms in the molecule, the process should be easier and even more conclusive than for chetoglobosin.

C) The structure of Triclinic Cobalt-tetraphenylporphyrin (with C. Kabuto).

This compound is a new triclinic form and is of interest for theoretical work on porphyrins because of anomalous magnetic properties. The results indicate a new and unique porphyrin conformation and theoretical considerations of the experimental parameters are in progress.

D) The structure of difluoro-diaziridino-benzoquinone (with T. Lee and C. Kabuto).

This relatively simple problem crystallographically was undertaken because the benzoquinone is a potential intermediate in anti-tumor drug synthesis and the substitution pattern could not be determined with certainty by other means. The structure has been determined and refined to an R-factor of 0.034.

E) The structure of a reaction product of glyoxal and dimethyl beta-ketoglutarate (with O. W. Adams and C. Kabuto).

This project continues work on an interesting and possibly valuable chemical method. X-ray data has been collected and solution of the phase problem in in progress.

F) Computer programs and theoretical work on quartet invariants (with T. Lee and C. Kabuto).

Programs have been developed to calculate quartet invariants for all space groups up to the orthorhombic system. The results of calculations have been compared with the actual results for A), B) and D). There is close accord between theoretical probabilities and actual results but, while the use of quartets for small molecules is obviously established, extension of their use to compounds of high molecular weight will require further theoretical calculations since probabilities have been shown to be low for large molecules. A possibly useful and readily calculated test has been deduced for calculation of negative quartets.

Publications

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4. Kapadia, G. J., Paul, B. D., Silverton, J. V., Fales, H. M. and Sokoloski, E. A. Melochinone, a novel quinolinone from Melochia tomentosa L. J. Amer. Chem. Soc. 97: 6814, 1975.

5. Akiyama, T. and Silverton, J. V. The structure of endo-tetracyclo(5,5,1,0,0)tridecane-4,8,12-trione, a system of four fused five-membered rings. Acta Cryst B31: 2336, 1975.

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7. Akiyama, T., Pedder, D., Silverton, J. V., Seeman, J. I. and Ziffer, H. A synthesis and X-ray structure determination of the photoproducts of a-homocholestan-3-one. J. Org. Chem. 40: 3675, 1975.

8. Akiyama, T. and Silverton, J. V. The crystal and molecular structure of a derivative of the terpene Spergulagen A. Acta Cryst B32: 58, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01006-05 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Characterization of Natural Matters

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Highet	Research Chemist	CH NHLI
OTHER:	F.-T. E. Chou	Visiting Fellow	CH NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

NMR: The effect on the C-13 chemical shifts of exchange of a hydroxy proton for deuterium has been studied and shown to allow counting the number of free hydroxy groups in an unknown material. The nuclear Overhauser effect (NOE) in dimethylpyrone has been shown to be concentration dependent.

Lipids: The study of spin-lattice and spin-spin relaxation mechanisms of the protons of high density lipoproteins (HDL) has been initiated.

Aromatic-Alicyclic tauteromerism: A series of model compounds have been synthesized to support structures proposed for the various tautomeric forms of dipyrrolidinophenol.

PHS-6040
(12-75)

Project Description

I. The Characterization of Materials by Nuclear Magnetic Resonance.

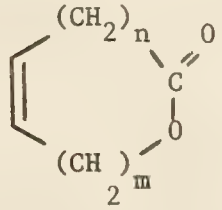
A. To aid in the characterization of alcohols by C-13 nmr, the effect of the conversion of ROH to ROD has been studied. Simply shaking the chloroform solution of the alcohol with deuterium oxide causes the α-carbon to be shifted upfield approximately 0.1 ppm, and allows free alcoholic groups in an unknown material to be counted. β-carbon atoms suffer smaller upfield shifts, dependent upon the number of protons borne by the atom.

B. Although the dependence of the nuclear Overhauser effect upon the distance separating the involved nuclei has been demonstrated, the results conflict with the well-established theory of the effect. Irradiating the methyl resonance of dimethylpyrone produces an enhanced signal for the olefinic proton with an unexpected concentration dependence which implies that a relaxation mechanism other than dipole-dipole interaction must be involved. It had not been supposed that spin relaxation mechanisms played a large role in such compounds, but such assumptions may have to be revised.

II. The Study of Lipoidal Materials

A. (With E. A. Sokoloski, LC, Brian Brewer, MDB, and James Osbourne, MDB). A study of the C-13 nmr properties of the proteins of high density lipoproteins has been initiated. Preliminary results from the apoprotein labelled with 1-C-13-S-carboxymethyl groups suggest that the dissappearance of the signals as the result of spin-spin relaxation may provide a monitor for the helix-random coil transition in this substance.

B. (With J. W. Wheeler, Howard University). The waxy material of molecular weight 224 from the anal scent gland of the hyena provides an interesting opportunity to develop methods of characterization of general utility for lipids. The C-13 spectrum of this material confirms the absence of methyl groups (and branching), and supports the formula I. However, to determine the position of the double bond will require



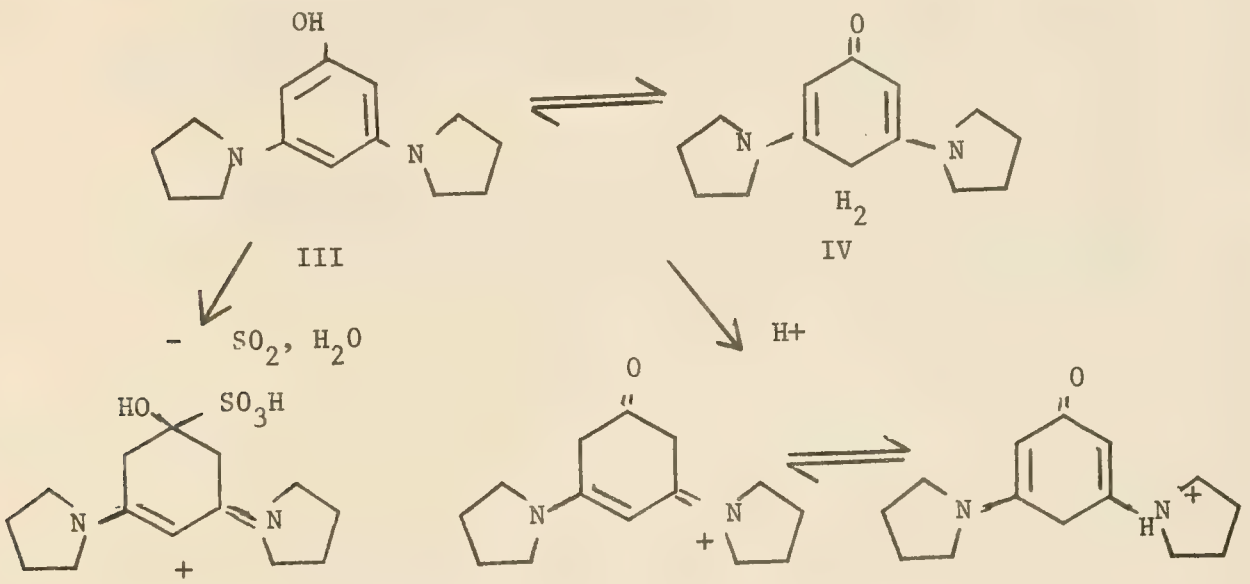
I, n+m = 11

II n = 5, m = 8

degradative methods. Such methods, suitable for use on microgram quantities, are now being sought, using ambrettolide as a paradigm. Although ambrettolide has been assigned formula II, examination of samples of this material from several sources suggests that various isomers may occur.

III The Study of Aromatic-alicyclic Tautomerism

Present interpretation of the tautomeric interconversion of dipyrrolidinophenol is summarized below:



In neutral solutions, the alicyclic form IV is favored by hydrogen bonding with proton-donating solvents; conversion of III to IV is incomplete in methanol and trifluoroethanol, but is complete in water.

We are now preparing model compounds of unambiguous structure to support these formulations. The solid phase infrared spectrum of the phenol suggests that both forms III and IV may be present in the crystal, a point which will be investigated by crystallographic methods with J. V. Silverton.

Publications

Highet, R. J., and Edwards, J. M. Analysis of the Carbon-13 NMR Spectrum of Phenalenone. J. Mag. Res. 17, 336-339, 1975.

Rice, K. C., Sharpless, N. W., Weiss, U., and Highet, R. J. The Reaction of Dimethyl β-Ketoglutarate with 1,2-Dicarbonyl Compounds. III. Exo-tetracyclo(5.5.1.$0^{2,6}$.$0^{10,13}$)tridecane-4,8,12-trione. Tetrahedron Lett. 3763-3766, 1975.

Rice, K. C., Weiss, U., Akiyama, T., Highet, R. J., Lee, T., and Silverton, J. V. Reaction of Dimethyl β-Ketoglutarate with 1,2-Dicarbonyl Compounds. IV. Formation of a Complex Tetracyclic Ring-System in Aqueous Solution at Room Temperature. Tetrahedron Lett. 3767-3770, 1975.

Smith, C. R., Madrigal, R. V., Weisleder, D., Mikolajczak, K. L., and Highet, R. J. Potamogetonin, a New Furanoid Diterpene. Structural Assignment by Carbon-13 and Proton Magnetic Resonance. J. Org. Chem. 41, 593, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01007-04 LC

PERIOD COVERED

July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Applications of Mass Spectrometry to Problems in Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. W. A. Milne	Research Associate	LC NHLI
OTHER:	G. J. Shaw	Guest Worker	LC NHLI
	G. J. Wright	Guest Worker	LC NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

A project to design a field desorption source for an MS-9 mass spectrometer has been continued. Useful field ionization mass spectra have been obtained with this source.

Th uses of stable isotopes in biomedicine have been explored. Syntheses of carbon-13 labelled alkanes, deuterium labelled serotonin and melatonin, and deuterium labelled imipramine and desipramine have been completed.

PHS-6040
(12-75)

Work has continued upon the development of a field desorption source and a source that works in the field ionization mode has been built. Its sensitivity is, however, not high and efforts are being made to deal with this problem. The design of a probe for use in field desorption has also caused some difficulties and a final design has yet to be arrived at.

Some effort has been made to equip the MS-902 mass spectrometer with a mass marker based upon the Hall effect. The device is working satisfactorily in the static mode but transfer of its signal to the recorder or to the computer during a scan has not yet been accomplished.

A series of projects involving stable isotopes have been completed. A synthesis of 1,12-$^{13}C_2$-n-dodecane has been carried out. This material is essential for studies of the fragmentation mechanism of n-alkanes upon field ionization.

The synthesis has been achieved of the widely sought after 1,1,2,2-tetradeuteroserotonin which is required as an internal standard for quantitation of serotonin by mass spectrometry. Some 10 g of this material has been prepared with a high degree of deuterium enrichment (98%) and it has been offered in small quantities to research workers in the biomedical area because it is unavailable commercially.

From this material, N-acetyl serotonin and melatonin (N-acetyl-O-methyl serotonin), both labelled with four deuteriums, have also been prepared in large quantity.

In a parallel project, imipramine and desipramine, labelled with deuterium, have been prepared for studies of the metabolism of these drugs that are being carried out in NIMH.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01008-05-LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Use of Digital Computing in Problems in Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. W. A. Milne	Research Chemist	LC NHLI
OTHER:	V. A. Vinton	Mathematician	LC NHLI
	S. R. Heller		MIDSD EPA

COOPERATING UNITS (if any)

EPA, NBS, ERDA, FDA, Agencies in U.K., Switzerland, Germany and Holland

LAB/BRANCH
Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION
NHLI-NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

The NIH-EPA Chemical Information System, comprising 13 different components, such as the mass spectral search system, is under systematic development in a project that involves the collaborative efforts of 10 agencies in the U.S. and Europe.

This multifaceted computer system is delivered to members of the international scientific community a fee-for-service basis via commercial networked computer systems and is currently being used by about 200 research scientists. Components of the Chemical Information System include programs and data bases in the areas of mass spectrometry, nmr spectrometry, structure determination by X-ray diffraction, X-ray crystal data, X-ray powder diffraction data, mass spectral bibliographic data, gas phase proton affinities, X-ray diffraction bibliographic data, and programs for NMR spectrum analysis, statistical analysis, and conformational analysis.

PHS-6040
(12-75)

Project No. Z01 HL 01008-05-LC

Project Description:

During the past year, the NIH-EPA Chemical Information System (CIS) has been placed upon a secure foundation that is being increasingly underpinned by further interagency and intergovernmental agreements.

Collaborating units in the CIS development now include NIH, EPA, NBS, ERDA, FDA, Department of Industry (U.K. Govt.), BASF and the German Cancer Research Foundation (Germany), ETH (Switzerland), and the Dutch Chemical Society.

Components of the CIS that are currently in the developmental phase at NIH or in the operational phase in the private sector include the mass spectral search system, the carbon-13 nmr search system, the X-ray crystal structure programs, the X-ray crystal data program, the X-ray powder diffraction data base, the sub-structure searching programs, the X-ray bibliographic data base, the mass spectrometry bibliographic data base, a gas phase proton affinity data base, programs for NMR analysis, statistical analysis, isotopic enrichment determination and conformational analysis of molecules in solution.

The financial feasibility in the private sector has been demonstrated for several of these components, operating upon a fee-for-service basis. The number of individual scientists around the world who are now using the CIS is approaching 200.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER 01 HL 01009-02 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies on the Enzymes Involved in the Activation of Human Plasma Kallikrein: Activation of Hageman Factor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. E. Webster	Senior Investigator	LC NHLI
OTHER:	J. V. Pierce	Senior Investigator	LC NHLI
	J. A. Guimaraes	NIH Postdoctoral Fellow	LC NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to investigate the enzymes and factors necessary for the activation of human plasma prekallikrein. Studies of the mechanism by which this enzyme is activated in human blood have clearly shown a relationship between the kallikrein-kinin system, the coagulation system and the plasminogen-plasmin system. The current data classifies human plasma kallikrein and its preferred substrate in plasma, high molecular weight kininogen, as two of intrinsic clotting factors and suggests that an unidentified factor (Factor W) is required for the full activation of Hageman factor and thus to initiate clotting, plasminogen activation and platelet aggregation.

PHS-6040
(12-75)

(This project was transferred from the Hypertension-Endocrine Branch as of July 1, 1975.)

Objectives: The kallikrein in human plasma is normally found as an inactive precursor prekallikrein. Our earlier studies and those of others had clearly shown that activation of this enzyme was required for activation of Hageman factor which then acts directly on its substrates, factor XI (PTA), plasminogen proactivator and prekallikrein, thus initiating the plasma proteolytic systems responsible for coagulation, clot lysis, kinin formation and possibly platelet aggregation. Activation of Hageman factor (HF) on a negatively charged surface was shown by us to require not only kallikrein but other unidentified factor(s). The purpose of this present study was to identify these factor(s) and to establish their role in the activation of Hageman factor.

Methods: Measurement of active Hageman factor adsorbed to a negative surface was determined using a radiochemical method employing (^{3}H)-TAMe as previously described (NHLI-75, 1973).

Major Findings: Our earlier studies had shown that neither plasmin nor plasminogen could activate crude Hageman factor adsorbed to a negative surface. Kallikrein, however, did generate some activity when added at twice the concentration found in normal plasma. Prekallikrein, on the other hand, failed to generate activity, although this same amount of prekallikrein could readily correct the defect found in Fletcher (prekallikrein) deficient plasma. It was suggested that unidentified factor(s) were required.

When various concentrations of kallikrein or prekallikrein were added to the crude Hageman factor or to Fletcher (prekallikrein) deficient plasma, it was again confirmed that the concentration of prekallikrein which would fully activate Fletcher plasma was incapable of activating the crude Hageman factor adsorbed to the negative surface. Even kallikrein could not fully activate this crude Hageman factor.

Shortly after these experiments, a new clotting defect was identified in several patients. These patients, like Hageman factor and Fletcher (prekallikrein) deficient patients showed a prolonged clotting time *in vitro* which could not be corrected by the addition of Hageman factor or prekallikrein. The new factor was subsequently identified as high molecular weight kininogen, the preferred substrate for plasma kallikrein. As in the studies with prekallikrein, the addition of this factor to the crude Hageman factor failed to form active Hageman factor at a concentration capable of fully activating the high molecular weight kininogen deficient plasma. However, unlike prekallikrein highly purified kininogen was capable of fully activating the Hageman factor if added in amounts 10 times that found in normal plasma.

The combination of prekallikrein and high molecular weight kininogen, at levels similar to those found in plasma, almost completely activated the crude Hageman factor. However, these same concentrations of prekallikrein and high molecular weight kininogen only partially activated pure Hageman factor. On the other hand, very high levels of high molecular weight kininogen could fully activate both crude and pure Hageman factor. These data suggested that preparations of crude but not pure Hageman factor contain a substance required for full activation of Hageman factor.

In other studies it was found that high molecular weight kininogen-freed of its kinin by digestion with kallikrein-retained its full ability to activate Hageman factor. Also, kinin itself is not involved in the activation process and cannot be substituted for high molecular weight kininogen. Similarly low molecular weight kininogen, the preferred substrate for glandular kallikreins, has no effect on the activating process.

Currently, attempts are being made to isolate the unidentified factor from various plasma fractions. Preliminary evidence suggests that it is destroyed by dilution of plasma. Also, the possibility is being investigated that the factor forms a complex with kininogen, thus explaining the ability of the latter to fully activate Hageman factor when added in excessive amounts. Current data suggests that filtration of normal plasma through low molecular weight kininogen-antibody-sepharose columns completely removes both high molecular weight kininogen and the new factor. Filtration of high molecular weight deficient plasma, on the other hand, does not remove the factor. Attempts to dissociate the factor from high molecular weight kininogen will be made.

Significance to Biomedical Research and Institute Program: The generation of kinins by plasma kallikrein has been implicated in a number of pathological conditions such as hereditary angioedema, gouty and rheumatic arthritis, asthma, pulmonary edema, reactions to blood transfusions, infection by pathogenic organisma, pancreatitis, etc. A complete understanding of the activation of this system which is intimately involved in both the formation and dissolution of clots, as well as the generation of kinins, could lead to the development of specific methods for blockade of this activation.

Proposed Course of Project: Continued investigation of the factors necessary for activation of Hageman factor.

Publications:

Miller, R. L., Webster, M. E. and Melmon, K. L: Interaction of leukocytes and endotoxin with the plasmin and kinin systems. J. Europ. Pharm. 33: 53-60, 1975.

Oh-ishi, S. and Webster, M. E.: Formation of prekallikrein activator and TAMe esterase activity by dilution of human plasma. In Pisano, J. J. and Austen, K. F. (Eds.) Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease. Fogarty Int. Center Proc. 27. U.S. Gov. Printing Office, Washington, DC (In press).

Webster, M. E. and Oh-ishi, S.: Activation of Hageman factor (Factor XII): requirement for activators other than prekallikrein. In Pisano, J. J. and Austen, K. F. (Eds.) Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease. Fogarty Int. Center Proc. 27. U. S. Gov. Printing Office, Washington, DC (In press).

Webster, M. E., Guimaraes, J. A., Kaplan, A. P., Colman, R. W. and Pierce, J. V.: Activation of Surface-Bound Hageman Factor: Pre-eminent role of high molecular weight kininogen and evidence for a new factor. Adv. Exp. Med. and Biol. (In press).

Newball, H. H., Keiser, H. R., Pisano, J. J. and Webster, M. E.: Effects of bradykinin on human airways. In Pisano, J. J. and Austen, K. F. (Eds.) Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease Fogarty Int. Center Proc. 27. U. S. Gov. Printing Office, Washington, DC (In press).

Meier, H. L., Scott, C. F., Mandle, R., Webster, M. E., Pierce, J. V. Colman, R. W. and Kaplan, A. P.: Activation of Human Hageman Factor: Interaction of Hageman factor with High Molecular Weight Kininogen, and Prekallikrein. Ann. N. Y. Acad. Sci. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01010-02 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biosynthesis of SRS-A in Monkey Lung

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	B. D. Davis	Predoctoral Investigator	LC NHLI
OTHER:	M. E. Webster	Senior Investigator	LC NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

SUMMARY OF WORK (200 words or less - underline keywords)

SRS-A (Slow Reacting Substance of Anaphylaxis) was first described in 1940 and shown to be released from monkey lung in 1966. Although SRS-A is well known as a mediator of bronchial asthma, the biochemical mechanism of its synthesis remains unknown. The precursors of SRS-A and the enzymes involved in its formation have not yet been described. The purpose of this study is to examine a number of compounds for their ability to increase the biosynthesis of SRS-A and for their possible incorporation into this biologically active compound.

PHS-6040
(12-75)

(This project was transferred from the Hypertension-Endocrine Branch July 1, 1975.)

Objectives: Although SRS-A (Slow Reacting Substance of Anaphylaxis) was first described in 1940 and considered to be one of the mediators of bronchial asthma, the biochemical mechanism of its synthesis remains unknown. The precursors of SRS-A and the enzymes involved in its formation have not yet been described. The purpose of this study is to examine a number of compounds for their ability to increase the synthesis of SRS-A and for their possible incorporation into this biologically active compound.

Methods: Monkey lung fragments (50-100 mg) were sensitized with human ragweed antisera, washed free of serum proteins and incubated for 1-60 min at 37° with varying concentrations of antigen E (5-5,000 ng). The biological activity of SRS-A is determined by bioassay utilizing the isolated guinea pig ileum in the presence of anticholinergic and antihistaminic agents. Histamine and arginine esterase are determined by radiochemical techniques described elsewhere (Webster et al., Ciencia e Cultura 26: 372, 1974; project report #Z01 HL 1945-03 HE, 1975).

Major Findings: Earlier studies (Z01 HL 1947-01 HE, 1975) had shown that monkey lung, like human lung, could be sensitized with human ragweed antibody, and would release, on addition of specific antigen, histamine, an arginine esterase and SRS-A. Further studies have suggested that the species from which the monkey lung was derived is of importance. For example, lung from the rhesus monkey usually generate more mediators than those derived from the Cercopithecus aethiops (African green). In addition the age of the animal influences the release of mediators, with lungs from younger animals releasing larger quantities of mediators. The reason for this latter difference is unknown but could involve the failure of human antibody to adsorb to monkey lung.

Attempts to develop a biochemical method for the detection of SRS-A activity have so far been unsuccessful. SRS-A probably inhibits arylsulfatase activity when p-nitrocatechol sulfate is used as substrate; but this assay cannot be employed in crude tissue supernatants because sulfate, phosphate and chloride ions also inhibit the enzymatic activity. Although elegant methods are available for the detection of thiol or disulfide groups, more detailed studies (Z01 HL 1946-02, 1976) indicated that SRS-A does not contain either of these chemical groupings. Thin-layer chromatography on DEAE-cellulose plates, like the silicic acid plates previously described (Z01 HL 1974-01 HE, 1975) resulted in essentially complete loss of activity, and conditions have not yet been found for the chromatographic separation of SRS-A. The best separation has been obtained by Sephadex LH-20 chromatography using 80% ethanol. However, methods must still be developed to remove or destroy an as yet unidentified compound or compounds which interfere with the bioassay.

A number of compounds were tested for their ability to alter the production of SRS-A in the monkey lung tissue supernatants. The sulfur-containing compounds, cysteine, sodium sulfide and methionine enhanced SRS-A production. Both oxidized and reduced glutathione were without effect on the synthesis of SRS-A and SO_4 at 10^{-3} M inhibited generation of mediators. Carboxylic acids such as succinic and α-ketoglutaric enhanced generation of SRS-A while mediator production was inhibited by malic acid. Both sodium sulfide and methionine required a preincubation period of 5 minutes for maximum formation of SRS-A. With succinic acid, however, increased amounts of SRS-A were generated whether the acid was added immediately after the addition of antigen or pre-incubated with lung tissue for a 5-minute period. These data would suggest that these two classes of compounds increase synthesis by different mechanisms. For example, the sulfur-containing compounds might be acting upon membranes and thus affect release of SRS-A while the carboxylic acids could be incorporated directly into the SRS-A molecule. Enhancement of SRS-A generation by sulfur-containing compounds is probably not the result of incorporation of these compounds, because both D-cysteine and L-cysteine are equally active in inducing increases in SRS-A synthesis. Further experiments involving incorporation of labeled compounds are in progress.

While succinate increases the production of SRS-A by two-fold, sodium sulfide doubles or trebles SRS-A activity. The combination of succinate and sulfide yields even greater amounts, again suggesting that the two compounds increase SRS-A activity by different mechanisms. Furthermore, preliminary evidence indicates that the SRS-A generated in the presence of sodium sulfide may have different chemical properties from that generated with succinic acid. For example, the SRS-A formed with sodium sulfide in the incubation medium, like Fractions II, III, and IV of human SRS-A, could not be extracted into chloroform; that formed in the presence of succinic acid like Fraction I, was readily extractable into chloroform. Further investigations will be required to determine whether monkey SRS-A, like the prostaglandins and human SRS-A's, represents a family of compounds.

Significance to Biomedical Research and Institute Program: Investigation into the mechanism of biosynthesis of monkey SRS-A should assist in the determination of the structure of SRS-A and in the development of antagonists to SRS-A which would be of therapeutic value in bronchial asthma.

Proposed Course of Project: Completion of current investigations into the biosynthesis of SRS-A.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01011-03 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structural Studies on SRS-A: Is SRS-A a Thiol or Disulfide?

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Hojima	NIH Postdoctoral Fellow	LC NHLI
OTHER:	M. E. Webster	Senior Investigator	LC NHLI
	B. D. Davis	Predoctoral Investigator	LC NHLI
	H. H. Newball	Asst. Prof. of Medicine	Johns Hopkins Univ. School of Medicine

COOPERATING UNITS (if any)

Johns Hopkins University School of Medicine

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

SUMMARY OF WORK (200 words or less - underline keywords)

SRS-A (Slow Reacting Substance of Anaphylaxis) is one of the mediators involved in bronchial asthma. The studies of other investigators and our own (Z01 HL 1946-02 HE, 1975) have provided evidence to suggest that SRS-A contains sulfur. The purpose of the present study is to determine whether SRS-A contains thiol or disulfide groups.

PHS-6040
(12-75)

(This project was transferred from the Hypertension-Endocrine Branch July 1, 1975.)

Objectives: SRS-A (Slow Reacting Substance of Anaphylaxis) is released from passivley sensitized human or monkey lung by addition of specific antigen and is one of the mediators involved in bronchial asthma. First described in 1940, SRS-A has not yet been isolated or its chemical structure determined. It is the purpose of the present study to determine whether SRS-A contains thiol or disulfide groups.

Methods: Human or monkey lung fragments (30-100 mg) were passively sensitized with ragweed antisera (1:40 dilution), washed free of serum proteins, and incubated for 15-30 min at 37° with specific antigen E (500 ng/tissue piece). Biological activity of SRS-A was measured by bioassay using the isolated guinea pig ileum in the presence of atropine and triprolidine. Thiols and disulfides were determined by their inhibition of the fluorescence of fluorescein mercuric acetate (FMA).

Major Findings: In our earlier studies (Z01 HL 1946-02 HE, 1975) human SRS-A's had been partially purified and separated into four biologically active fractions, only one of which lost its activity when incubated with arylsulfatase (Type H-1) at pH 4.5. Nevertheless, all four fractions inhibited the arylsulfatase activity when p-nitrocatechol sulfate was used as substrate. These data were in agreement with the possibility that the SRS-A's contain a sulfur group.

As these studies were being concluded, it was found that highly purified Fraction I could be extracted into chloroform from water solution at pH 4.5, while Fractions II, II and IV remained in the water layer. These findings suggested that a simple rapid procedure could be devised for the separation of Fraction I from crude ethanol extracts. Chloroform extraction of ethanol extracts gave a recovery of 18-36% of the SRS-A in the chloroform layer an amount which agreed well with previous estimates for Fraction I. When the ethanol extracts were incubated with 0.1 M NaOH for 30 min at 37° at a ratio of alkali to ethanol extract 10 times that previously employed, the yield of Fraction I in the chloroform layer increased to 46-75%, indicating that this alkali treatment had converted the major portion of the other fractions into Fraction I. Investigation of the conditions necessary for extraction of Fraction I showed that it could be extracted into chloroform at pH 4.5 or pH 6.5 but not at pH 8.5, suggesting that SRS-A is a weakly acidic compound.

As the SRS-A's like thiols and disulfides, are sensitive to light and susceptible to oxidation, the possibility that SRS-A contains a thiol was examined. Initially it was shown that the addition of antigen to passively sensitized human or monkey lung tissue caused the release of both a thiol and a disulfide as determined by their ability to inhibit the fluorescence of fluorescein mercuric acetate (FMA). The inhibition curve shown by the lung compounds differed from that given by cysteine, cystine, reduced

glutathione or oxidized glutathione. However, following incubation with alkali and extraction with chloroform only 10% of the thiol and disulfide were found in the chloroform layer together with 50-75% of the biological activity of SRS-A. These data suggested that the major portion of the thiol and disulfide present in these lung extracts was not due to SRS-A but did not eliminate the possibility that the residual activity found in the chloroform layer was due to SRS-A.

Attempts were made to reduce the disulfide to its thiol and to assess the effect of these procedures on the biological activity of SRS-A. Most disulfide bonds are cleaved in alkali. Incubation of the ethanol extracts with 1 M NaOH for 30 min at room temperature and trapping of the resulting thiol by binding to FMA did cleave the disulfide but had no effect on the biological activity of SRS-A. These data would indicate that if SRS-A is a disulfide, both the reduced and oxidized forms had equivalent biological activity.

Sodium borohydride is another method widely used for the reduction of disulfides to thiols. Under conditions suitable for the cleavage of cystine and oxidized glutathione, the dithiol in the ethanol extracts was cleaved and the SRS-A activity was lost. However, further examination of this reaction showed that the biological activity of SRS-A was extremely labile under the conditions of reduction and could be lost during 1 min incubation when the disulfide bond had not yet been cleaved.

DTE (1,4 dithioerythritol) is also an excellent reductant for disulfides and reduced disulfides by thiol-disulfide exchange. However, incubation of the ethanol extracts with DTE did not alter the biological activity of SRS-A. In general these data did not support the hypothesis that SRS-A was a disulfide but neither did they eliminate the possibility that both the oxidized and reduced forms had biological activity.

However, the observation that SRS-A in the ethanol or chloroform extracts can be filtered through an agarose column which binds thiols without loss of biological activity make it unlikely that SRS-A is a thiol compound. This column was prepared by binding glutathione to agarose and in turn linking the glutathione to 2-pyridylsulfide (Brocklehurst and Carlsson, Biochem. J. 133:573, 1973). Thiol compounds bind to the column by thio-disulfide exchange with the 2-pyridylsulfide. However the SRS-A present in either the ethanol extracts or chloroform soluble fraction did not bind to the column and the activity could be completely recovered in the filtrate. Even prior treatment with DTE failed to form an SRS-A which could be adsorbed to the agarose thiol coupler.

In one additional experiment, attempts were made to desulfurize SRS-A by treatment with Raney nickel. However, when the ethanol extracts or chloroform soluble SRS-A was treated with Raney nickel under conditions suitable for desulfurization of oxidized glutathione, no desulfurization of the thiol or disulfide present in these extracts occurred. However, as with

reduction with sodium borohydride, the biological activity of SRS-A was easily destroyed.

It appears unlikely, therefore, that SRS-A contains either a thiol or disulfide grouping. Nevertheless the data have provided further information concerning the chemical nature of SRS-A. The partition of SRS-A into chloroform would suggest a weakly acidic compound and would argue against the presence of either a free carboxylic acid or a sulfate group. In addition to its known acid lability, sensitivity to light and susceptibility to oxidation SRS-A is also susceptible to reduction. The ease with which sodium borohydride can destroy the biological activity would suggest an aldehyde, ketone, sulfoxide or sulfone and experiments aimed at eliminating or confirming these possible structures should be conducted.

Significance to Biomedical Research and the Program of the Institute: SRS-A is one of the few remaining mediators of bronchial asthma whose chemical structure remains unknown. The determination of its structure would greatly aid the development of antagonists to this vasoactive substance which may be clinically useful.

Proposed Course: This project will be discontinued.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01012-03 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Amino Acid Sequence Determination of Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. C. Krutzsch	Staff fellow	LC NHLI
OTHER:	J. J. Pisano	Chief, Sec. on Physiol Chem.	LC NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

A dipeptidyl aminopeptidase method for polypeptide sequencing using gas chromatography-mass spectroscopy for dipeptide identification has been further developed. A new dipeptidyl aminopeptidase has been discovered which cleaves amide bonds involving the imino group of proline. When used in conjunction with the previously reported DAP-I and IV, it should be possible for the first time to confidently degrade any polypeptide. The use of the combined DAP enzymes and our GC-MS method for dipeptide analysis appears to be a superior sequencing technique with sensitivity and speed as the major assets.

PHS-6040
(12-75)

Project No. Z01 HL 01012-03 LC

(This project was transferred from the Hypertension-Endocrine Branch July 1, 1975.)

Objective: To increase the sensitivity and speed of polypeptide sequencing through the use of dipeptidyl aminopeptides (DAP) to degrade polypeptides and gas chromatography-mass spectrometry (GC-MS) to determine the released dipeptides.

Methods Employed:

1) DAP digestion of the polypeptide and its single Edman-degraded congener.

2) GC-MS of the volatile dipeptide derivitive mixtures for separation and identification of the dipeptides.

3) Combination of the two sets of dipeptide fragments to yield the structure of the polypeptide.

Major Findings: The DAP method of polypeptide sequencing requires a combination of DAP's to effect the degradation of all possible polypeptides. This need for several enzymes occurs because enzyme DAP-I (used for most digestions) lacks the ability to cleave amide bonds involving either the carboxyl or the imino group of proline. This requirement is met through the application of two other DAP's, DAP-IV and V. DAP-IV cleaves dipeptide fragments with amide bonds involving the carboxyl group of proline and DAP-V amide bonds involving the imino group of proline. Both of these enzymes, however, lack the otherwise broad specificity of DAP-I. DAP-V is a new enzyme discovered in our laboratory which promises to extend the DAP/GC-MS method to all polypeptides.

The purification of DAP-V requires separation from a host of interfering enzymes, including monopeptides, endopeptidases and dipeptidases, as well as material causing extraneous GC peaks. Accordingly, a major part of the past years' work has been devoted to purification of this enzyme, confirming the enzymes specificity and developing a large scale method. The purification of the enzyme has entailed trying numerous methods, including CM and DEAE ion exchange, hydroxyapetite, gel filtration, isoelectric focusing, disc gel electrophoresis, and affinity chromatography. A set of parameters has been defined for every step used.

Cleavage of dipeptides from the imino side of proline was investigated using the model peptides gly-pro-pro-ala, gly-pro-pro-pro, pro-ala-pro-pro and val-ala-pro-ala. The polypepide gly-pro-gly-gly-pro-ala was also successfully digested with a combination of DAP-IV and V, yielding gly-pro, gly-gly, and pro-ala. Because all contaminating dipeptidase enzyme had not been removed, the corresponding amino acids were also present. The need for a free amino group was confirmed with the CBZ-amino blocked analog of this polypeptide. When this compound was subjected to digestion, no dipeptides or amino acids were obtained, ruling out the possibility that digestion was aided by either an endopeptidase or a dipeptidyl carboxypeptidase.

Similarly, DAP-V, in combination with DAP-I digested bradykinin (arg-pro-pro-gly-phe-ser-pro-phe-arg), as indicated by the appearance of phe-ser. Arg-pro, pro-gly and pro-phe were digested further to their respective amino acids by contaminating dipeptidase. Blanks of bradykinin or bradykinin plus DAP-I did not yield any detectable phe-ser or amino acids. Angiotensin II was also digestable with the combination of DAP-I and V. The length of the polypeptide chain appears to pose no blockade to DAP-V either, as gly-pro was successfully cleaved off of a thirty residue polypeptide chain whose sequence began with gly-pro-hypro-.

The major steps required for the large scale production of DAP-V have been determined. As currently conceived, the preparation involves five steps: 1) homogenization of hog kidney followed by Triton X-100 extraction of the particulate matter; 2) ammonium sulfate precipitation; 3) CM-cellulose ion exchange; 4) gel filtration; and 5) affinity column chromatography.

Other areas of the DAP/GC-MS method of polypeptide sequencing were also investigated. The gas chromatography step has been improved to the point that only arginine-containing dipeptides require direct probe introduction into the mass spectrometer. Previously, some dipeptides containing gln, asn or his also required direct probe introduction for identification. If the guanido group of arginine is converted to the dimethyl pyrimidine moiety with acetyl acetone, these dipeptides also are elutable from the gas chromatograph.

Another improvement was to change the method of producing the polypeptide overlaps. The previous procedure involved the performance of a single Edman degradation. However, when small quantities of polypeptide were present, this sometimes resulted in objectionable amounts of GC background-forming material, extractive losses (giving in turn loss in sensitivity), and difficulty in identifying the amino terminal amino acid. The new procedure involves the addition of one amino acid residue, 1-α-amino butyric acid (1-α-aba). Because 1-α-amino butyric acid is not one of the common twenty amino acids, the N-terminal amino acid X is identified as 1-α-aba-X in the same GC-MS step used for identification of the overlapping dipeptides. There is no extractive losses and GC background-forming byproducts are virtually eliminated.

Signficance to Biomedical Research and the Program of the Institute: The work described above will significantly increase the scope, utility and sensitivity of polypeptide sequencing. The method will help to provide the required sensitivity and confidence in the elucidation of the structures of small quantities of biologically important polypeptides, such as enzymes transplantation antigens, hormones, cell growth factors, etc.

Proposed Course: Future work will center on completion of the large scale preparation of the new enzyme, DAP-V. The new sequencing methodology will be extensively applied to document its utility.

Publications:

Krutzsch, H. C. and Pisano, J. J.: The use of Dipeptidyl Aminopeptidase (DAP) and Gas Chromatography-Mass Spectrometry (GC-MS in Polypepide Sequencing. In Walter, R. and Meienhofer, J. (eds.): Peptides: Chemistry, Structure and Biology, Ann Arbor, Mich., Ann Arbor Science Publishers, 1975, pp. 985-900.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01013-04 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Histamine, Prostaglandins and Esterases from Human Lung

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. E. Webster	Senior Investigator	LC NHLI
OTHER:	H. Takahashi	NIH Postdoctorate Fellow	LC NHLI
	H. H. Newball	Asst. Prof. of Medicine	Johns Hopkins Univ. School of Medicine

COOPERATING UNITS (if any)

Johns Hopkins University School of Medicine, Baltimore, Maryland

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Histamine, SRS-A (Slow Reacting Substance of Anaphylaxis), arginine esterase, plasminogen activator and prostaglandins are released from human lung during passive sensitization by the addition of specific antigen. The conditions necessary for their release and the role of these compounds in allergic asthma in man was studied. The data provide a possible explanation for the well known aspirin sensitivity seen in about 10% of adult asthmatics.

PHS-6040
(12-75)

(This project was transferred from the Hypertension-Endocrine Branch July 1, 1975.)

Objectives: The passive sensitization of human lung fragments as first described by Parish (Nature 212:738, 1967) provides a laboratory model for the study of allergic asthma in man and permits investigation of the role of mediators and modulators in this immediate type allergy. The purpose of this study was to identify mediators released from human lung during antigen-antibody interaction; to determine the mechanism of their release; to assess the role of these compounds in the induction of bronchial asthma in man; and to provide a rational basis for the development of new drugs for therapy.

Methods Employed: Human lung obtained during surgery is cut into fragments (30-100 mg), sensitized with sera obtained from individuals allergic to ragweed, washed free of serum proteins and incubated with a preparation of pure antigen E (5-500 ng). Release of arginine esterase and histamine were determined by sensitive radiochemical assays developed in this laboratory (Beaven et al., Clin. Chim. Acta 32:67, 1971; Webster et al., PHS-NHLI, Z01 HL 1945-03 HE, 1975).

Major Findings: Our earlier studies had shown that human lung tissue passively sensitized with homologous ragweed IgE antibody, releases, upon exposure to specific antigen, histamine, SRS-A (Slow Reacting Substance of Anaphylaxis), an arginine esterase and prostaglandin E's. Prostaglandin F's and plasminogen activator, on the other hand, showed minimal increases. The addition of arachidonic acid (0.5×10^{-3} M), a substrate for prostaglandin synthetase, to tissue supernatants inhibited the release of histamine and SRS-A but, as expected, the yield of prostaglandin E's increased by 20-fold and of prostaglandin F's by 3-eold. Indomethacin (5 µg/ml) inhibited the formation of the prostaglandins but did not alter the release of histamine. Sensitized human lung tissue either in the presence or absence of antigen metabolized only 20% of added prostaglandin E_1 or $F_{2\alpha}$.

As prostaglandin E's constrict and prostaglandin F's relax human bronchi, it appears unlikely that the prostaglandins would have a direct mediator effect in bronchial asthma. They could act as modulators of this response by altering levels of cyclic AMP and thus increasing or decreasing mediator release (Tauber et al., J. Immunol. 111:27, 1973). These investigators have shown that the exogenous addition of prostaglandins do indeed increase or decrease mediator release and we have found a similar effect with endogenously synthesized prostaglandins. These data provide an explanation for the well known aspirin sensitivity seen in about 10% of adult asthmatics. It may be that these individuals are producing relatively large amounts of prostaglandins, sufficient to increase levels of cyclic AMP so that mediator release in inhibited. On the other hand, the occasional patient who shows striking relief of symptoms following injection of aspirin may be synthesizing such small amounts of prostaglandins that levels of cyclic AMP are decreased, thus causing an increase in mediator release.

Project No. Z01 HL 01013-04 LC

Significance to Biomedical Research and the Program of the Institute: The identification of mediators released from human lung by antigen-antibody reaction coupled with an investigation of the mechanism of their release and the compounds which inhibit this release should provide a rationale for the development and use of therapeutic agents for bronchial asthma.

Proposed Course: This project will be discontinued.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 H1 01014-06 LC

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Peptide Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Pisano	Chief, Sec. on Physiol. Chem.	LC NHLI
OTHER:	H. Nakamura	Visiting Fellow	LC NHLI
	C. L. Zimmerman	Chemist	LC NHLI
	E. Appella	Medical Officer (Biochemist)	LCB NCI

COOPERATING UNITS (if any)
Laboratory of Cell Biology, NCI

LAB/BRANCH
Laboratory of Chemistry

SECTION
Physiological Chemistry

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0

SUMMARY OF WORK (200 words or less - underline keywords)

All 20 common PTH amino acids can be separated in less than 20 min using a 25 cm x 4.6 mm Zorbax ODS column. A micro method has been developed for the dedection of peptides, amino acids and other substances with primary amino groups on TLC plates using Fluorescamine

PHS-6040
(12-75)

(This project was transferred from the Hypertension-Endocrine Branch July 1, 1975.)

Objectives: To develop necessary methodology for the micro characterization of proteins, peptides and amino acids.

Methods employed: High performance liquid chromatography (HPLC) spectrophotofluorometry, thin-layer chromatography (TLC) automated and manual Edman degradation of polypeptides.

Major findings: With recent advances in HPLC column technology, we have resolved in less than 20 min. all 20 common PTH amino acids with a 25 cm x 4.6 mm column of Zorbax ODS (Dupont Instruments).

A micro (0.03 µg) method has been developed for the detection of polypeptides, amino acids and other substances wtih primary amino groups on TLC plates, using Fluorescamine.

A new type of TLC plate was evaluated for the detection of UV-absorbing derivatives of peptides and amino acids. The utility of the plate was demonstrated in the analysis of PTH amino acids obtained in an automated Edman degration.

Significance to Biomedical Research: Most labs involved in amino acid sequence analysis of polypeptides have a critical need for greater sensitivity and speed of analysis. The methods just reported should help to satisfy this need.

Proposed Course of Project: To demonstrate the value of the new methods in the amino acid sequence of polypeptides. To apply the methods in the discovery of new biologically active peptides.

Publications:

1. Nakamura, H. and Pisano, J. J. Sensitive fluorometric assay for proteins: Use of fluorescamine and membrane filters. Arch. Biochem. Biophys. 172:102-105, 1976.

2. Nakamura, H. and Pisano, J. J. Reaction of tryptophane, tryptamines, and some related indoles with fluorescamine: Unique fluorescence in strong acid. Arch Biochem. Biophys. 172:98-101, 1976.

3. Yoshida, H., Zimmerman, C. L., and Pisano, J. J. High performance liquid chromatography and droplet countercurrent chromatogaphy in the peptide laboratory. In Walter, R and Meinenhofer, J. (Eds.): Peptides: Chemistry, Structure and Biology. Ann Arbor, Ann Arbor Science, 1975, 955 pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01015-05 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization of Villikinin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John J. Pisano	Chief, Sec. on Physiol. Chem.	LC NHLI
OTHER:	Carl Zimmermann	Biochemist	LC NHLI
	Eszter Kokas		Univ. of N.C. Medical School

COOPERATING UNITS (if any)

Dept. of Physiology, University of North Carolina
School of Medicine, Chapel Hill, NC

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

SUMMARY OF WORK (200 words or less - underline keywords)

Porcine villikinin like canine villikinin is a 2000 m.w. polypeptide which has the unique action of causing a relatively prolonged pumping of dog intestinal villi when injected I.V. Villikinin has been partially purified.

401

PHS-6040
(12-75)

Project No. Z01 HL 01015-05 LC

(This project was transferred from the Hypertension-Endocrine Branch July 1, 1975.)

Objectives: To isolate and determine the structure of villikinin, a putative gastrointestinal hormone obtained from intestinal mucose which has a specific stimulant action on intestinal villons motility.

Methods Employed: Canine and porcine intestinal mucose samples were extracted with trichloroacetic acid and the extracts applied to Dowex 50, Bio Gel P-4 and P-10 columns. Further purification was achieved using droplet countercurrent chromatography. The activity of the fractions was determined by bioassay in vivo, by the usual method of noting the increase in intestinal villons motility.

Major Findings: Extracts of mucosea from 75 pigs contained 7000 units of villikinin and weighed 375 mg. After Bio Gel chromatography and droplet countercurrent chromatography, 700 units were recovered weighing 9.5 mg. Porcine villikinin like canine villikinin is a 2000 MW polypeptide.

Significance to Biomedical Research and the Program of the Institute:

Villikinin appears to be a specific hormone which causes intestinal villi to contract. This pumping of the villi promotes lymph flow and presumably absorption of nutrients from the GI tract.

Proposed Course: To further purify and determine the structure of porcine villikinin.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01016-06 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Clinical Biochemistry of the Kallikrein-Kinin System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Pisano	Chief, Sec. on Physiol. Chem.	LC NHLI
	J. Corthorn	Visiting Fellow	LC NHLI
	J. Pierce	Research Chemist	LC NHLI
	K. Yates	Chemist	LC NHLI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	

SUMMARY OF WORK (200 words or less - underline keywords)

A kallikrein inhibitor complex has been isolated from human urine. Trypsin displaces the inhibitor and activates kallikrein but is itself inactivated. Urines from subjects with a congenital deficiency of kininogen contain little or no kinins. Normal human urine contains kininogen. Kinins liberated from this kininogen are 50-100% the normal kinin level.

PHS-6040
(12-75)

Project No. Z01 HL 01016-06 LC

(This project was transferred from the Hypertension-Endocrine Branch July 1, 1975.)

Objectives: To isolate and identify an esterase in human urine which is released after treatment with trypsin. To establish that trypsin binds an inhibitor rather than activating a precursor. To characterize the inhibitor. To develop procedures for the assay of components of the kallikrein-kinin system. To establish the role of the kallikrein-kinin system in health and disease.

Methods employed: Radioimmunoassay of kininogen and kinins. Radiochemical assay of prekallikrein and kallikrein. Enzymatic digestion of plasma and urine with trypsin. Ion-exchange chromatography, gel-filtration.

Major findings: Purification of a factor from human urine which releases an esterase upon treatment with trypsin was achieved using a linear gradient from 0.1 to 0.3M phosphate pH 6.0 in a DEAE-cellulose column which separated it from kallikrein. This factor, after treatment with trypsin, releases kinins (rat uterus bioassay) from boiled citrated plasma or purified kininogen B2α and it shows the same ratio of kininogenase to esterase activity as standard human urinary kallikrein. Increasing amounts of the untreated factor inhibits trypsin's esterase activity. The data indicate that the factor is kallikrein bound to an inhibitor which has a greater affinity for trypsin. The estimation of the molecular weight by gel filtration of this inhibitor-kallikrein complex is 50,000 whereas the apparent molecular weight of free kallikrein is 37,500. Purified kininogen B2α preincubated with urinary kallikrein doubles its esterase activity.

Urine from two subjects with congenital deficiencies of kininogen contained <1% and <0.1% the normal kinin levels which correlated with their kininogen which was 9% and <1% normal.

Normal human urine contains kininogen. Kinins liberated from this kininogen are 50-100% the level of free kinins in normal urine. A subject with Bartter's syndrome who excreted very little kinins had no detectable kininogen in her urine.

Significance to Biomedical Research and the Program of the Institute: Characterization and assay of the components of the kallikrein-kinin system is essential to elucidating its apparent important role in the kidney and in the regulation of blood pressure.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01017-04 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Metabolism of Prostaglandin A

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. M. Cagen	Staff Fellow	LC NHLI
OTHER:	J. J. Pisano	Chief, Sec. on Phys. Chem.	LC NHLI
	H. M. Fales	Chief, Lab. of Chem.	LC NHLI
	R. E. Bowden	Clinical Associate	HEB NHLI
	R. M. Zusman	Clinical Associate	HEB NHLI
	M. P. Peyton	Chemist	LC NHLI

COOPERATING UNITS (if any)

Hypertension-Endocrine Branch, NHLI

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Reduction of the 9-keto group of the glutathione conjugate of PGA_1 was catalyzed by red blood cell suspensions, lyzed red cells, and purified 9-hydroxyprostaglandin dehydrogenase. The latter reaction is 24,000 times faster than the corresponding reduction of PGE_1.

PGA_1 infused into the canine renal artery appeared in urine in the form of polar conjugates with the chromatographic properties of cysteine and N-acetyl cysteine PGA_1. PGE_1 and $PGF_{1\alpha}$ were converted to less polar metabolites in similar experiments.

Evidence was obtained for the occurrence in human urine of a conjugate of PGA_2.

PHS-6040
(12-75)

This project was transferred from the Hypertension-Endocrine Branch June 1, 1975.)

Objectives: To study the metabolism of Prostaglandin A (PGA) and to investigate the presence of metabolites of PGA in human urine as evidence of its natural occurrence in man.

Methods: Metabolites of PGA were purified by thin-layer and column chromatography and analyzed by mass spectrometry.

Major Findings: PGA_1 is converted by whole human blood and by suspensions of red blood cells to two glutathione (GSH) conjugates. The first of these ($GSH\text{-}PGA_1$) is identical to the conjugate formed by nonenzymic reaction of GSH with PGA_1. This reaction is also catalyzed by homogeneous proparations of glutathione S-transferase from human and rat liver. Glutathione S-transferase is present in human red cells. The second conjugate ($RGSH\text{-}PGA_1$) differs from $GSH\text{-}PGA_1$ in that the 9-keto group of the prostaglandin moiety has been reduced to the corresponding alcohol. $RGSH\text{-}PGA_1$ was also prepared synthetically by treating $GSH\text{-}PGA_1$ with sodium borohydride.

PGA_2 is converted to a similar mixture of glutathione conjugates. Other prostaglandins (PGE_1, $PGF_{2}\alpha$) are not metabolized by human red cell suspensions.

At physiological concentrations of PGA_1 (1 pmole/ml), RGSH-PGA, is the major product of red cell metabolism. Even at the higher concentrations employed in preparative experiments (0.3 μmole/ml) $RGSH\text{-}PGA_1$ represented 60% of the glutathione conjugate formed. Reduction of the 9-keto group must follow addition of GSH since reduced PGA_1 is not susceptible to conjugate addition reactions. Synthetic $GSH\text{-}PGA_1$ is converted to $RGSH\text{-}PGA_1$ by lyzed red blood cells and by red blood cell suspensions, though the latter do not cause the corresponding conversion of PGE_1 to $PGF_{1\alpha}$. Purified chicken heart and chicken kidney 9-hydroxyprostaglandin dehydrogenase (9-PGDH) catalyzes conversion of $GSH\text{-}PGA_1$ to $RGSH\text{-}PGA_1$ much more rapidly than the corresponding reduction of PGE_1. At physiological concentration (10^{-8} m) the reduction of $GSH\text{-}PGA_1$ is 24,000 times more rapid than the reduction of PGE_1 with the chicken heart enzyme and 5700 times more rapid with the chicken kidney preparation. $GSH\text{-}PGA_1$ is the best substrate so far described for this enzyme.

No evidence was obtained for the conversion of PGA_1 or A_2 to the corresponding 15-keto or 9-hydroxy derivatives by human red cells despite the presence in these cells of 9- and 15-PGDH (Kaplan et al. Arch. Bioch. Bioph. 167, 287 (1975)). Apparently the rapid conversion of PGA to GSH-PGA precludes other modes of metabolism in these cells.

It was desirable to have on hand authentic 9-hydroxy PGA_1 as a standard for mass spectrometry to aid in analyzing red cell metabolic products. This was prepared from PGA_1 by a Meerwein-Ponndorf reduction since reduction with metal hydrides yielded only 10,11 dihydro-9-hydroxy PGA_1.

When [^{3}H]PGA_1 is injected into the canine renal artery, 40% of the radioactivity appears in the renal venous effluent (within 1 minute of injection) and 20% in the urine (within 30 minutes of injection). Metabolites in the plasma are less polar than PGA_1 and are most likely the 15-keto and 13,14 dihydro-15-keto derivatives of PGA_1. The urine metabolites were more polar than PGA_1. At least 3 such metabolites could be distinguished by thin-layer chromatography, together comprising 75% of the radioactive material in urine. No GSH-PGA or RGSH-PGA_1 was observed. However, the two principal metabolites appear to be the cysteine and N-acetylcysteine adducts of PGA_1, (which have been in part reduced at the 9-keto position); these are the expected renal metabolites of a glutathione conjugate. The remaining radioactive material in urine is distributed between PGA_1 and a less polar metabolite.

In contrast to PGA_1, PGE_1 and $PGF_1\alpha$ are not converted to polar metabolites when injected into the renal artery. The urinary metabolites of these compounds appear to be the 15-keto and 13,14 dihydro 15-keto derivatives. At least in the dog, the kidney does not seem to be the site of ω-oxidation of prostaglandins.

The experiments with dog kidney suggests that if PGA is produced renally in humans, it would appear in urine largely in conjugated form(s).

Such conjugates can be converted by saponification to PGB (when the 9-keto group is intact). Samples of human female urine, from which unconjugated prostaglandins had been removed by extraction, were treated with KOH and the newly released immunoreactive PGB (iPGB) measured by radioummunoassay. Saponification consistently releases 1-2 pmoles/ml of iPGB which coelutes with authentic PGB during silicic acid chromatography. Treatment of human subjects with prostaglandin synthetase inhibitors reduced the levels of iPGB released from urine samples in proportion to that of unconjugated PGE_2 in the same urine.

The prostaglandins synthesized by growing cultures of rabbit renal interstitial cells (the cell-type chiefly responsible for prostaglandin synthesis in kidney) were analyzed by gas chromatography-mass spectrometry. PGE_2 is the principal prostaglandin produced by these cells together with smaller amounts of PGE_1, PGA_2, and PGA_1 (the latter two prostaglandins may arise by nonenzymic dehydration of PGE_2 and PGE_1). A novel C_{22} prostaglandin, dihomo PGE_2 was also observed in the culture medium.

Variable recoveries were observed when known amounts of PGA_2 were added to and extracted from plasma. These data are now thought to be due to: 1) poor solubility of prostaglandins in the aqueous buffer used for radioimmunoassay. Taking up dried samples in ethanol to equal 10% of the final volume before added aqueous buffer improves the data as does diluting and pipetting standards and unknowns at room temperature rather than at 4°. 2) poor stability of PGA_2 in aqueous buffer. The standards should be prepared frequently from methanol solution.

In the course of these investigations, the possibility that PGA was converted during silicic acid chromatography to a compound with different affinity for the PGA antiserum was studied. The evidence did not support this theoretical explanation of the variable recovery data.

Significance for Biomedical Research:

PGA_1 is a potent vasodilator and natriuretic substance that has been used experimentally for the treatment of hypertension; it has the unique advantage of maintaining high rates of sodium excretion as blood pressure is lowered. A limitation on the use of this drug has been the requirement that it be administered by continuous intravenous infusion. Understanding the metabolism of PGA_1 may permit the development of analogs less rapidly cleared from the circulation.

PGA_1 differs from other prostaglandins in being susceptible to conjugation with glutathione. This is the principal mode of metabolism in human blood. Alteration of PGA_1 to make it less susceptible to conjugate addition e.g. by selective reduction of the 9-keto group or of the 10,11 duble bond might increase its half-life in human circulation. A method for achieving the former reduction was developed.

It has been proposed that PGA_2 is a naturally occurring renal hormone, though attempts to detect its production by mammalian kidney have been unsuccessful. This could have been due in part to the susceptibility of PGA_2 to conjugation reactions. Experiments with dog kidney suggest tht renally produced PGA_2 would be excreted principally in conjugated forms. The occurrence of a conjugate(s) of prostaglandin in human urine suggest that PGA might indeed be produced in human kidney.

The observation that GSH-PGA_1 is 24,000 times more reactive with 9-PGDH than PGE_1 raises the question whether it, or some other PGA conjugate, might not be the natural substrate for this widely distributed enzyme. At this time no data exists on the occurence of prostaglandin conjugates in mammalian tissues.

Proposed Course:

1) The canine renal metabolites will be further analyzed by GC-MS after treatment with Raney nickel and CNBr to determine if the prostaglandin moiety has undergone alterations in addition to the conjugation reaction.

2) The prostaglandin liberated from human urine by saponification will be determined by HPLC and GC. This is needed to confirm the results of radioimmunoassy.

3) A method for separating PGB_2 from PGB_1 and from other prostaglandin metabolites will developed and incorporated into the radioimmunoassay

procedure for urinary prostaglandins. This will give the method added specificity and assure that only renally produced prostaglandins are being measured.

4) The reaction of GSH with PGA can be exploited to purify PGA_2 from human plasma greatly increasing the sensitivity and specificity of the radio-immunoassay procedure for this substance. The method will be employed to determine the levels of PGA_2 in human plasma with greater confidence.

Publications:

1. Cagen, L. M., Pisano, J. J., Ketley, J. N., Habig, W. H., and Jakoby, W. B. The conjugation of prostaglandin A_1 and glutathione catalyzed by homogeneous glutathione S-transferases from human and rat liver. BBA 398, 205 (1975).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01018-19 LC

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Kallikrein-Kininogen-Kinin System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. V. Pierce	Research Chemist	LC NHLI
OTHER	J. A. Guimaraes	Visiting Fellow	LC NHLI
	J. J. Pisano	Chief, Sec. on Physiol.Chem.	LC NHLI
	M. E. Webster	Research Chemist	LC NHLI
	A. P. Kaplan	Medical Officer	LCI NIAID
	R. W. Colman	Professor	Univ. of PA

COOPERATING UNITS (if any)

Allergic Diseases Section, LCI, NIAID

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	

SUMMARY OF WORK (200 words or less - underline keywords)

Human high molecular weight(HMW) kininogens are now established as a factor required for surface activation of Hageman factor. B4β or B4γ kininogen, used at 10% the concentration of HMW kininogen in normal plasma, completely corrects the clotting and other defects of Williams plasma (Kininogen-deficient). The B4 kininogens also enhance the ability of active Hageman factor to activate its three natural substrates, plasma thromboplastin antecedent, and prekallikrein), also the ability of Hageman factor fragments to activate prekallikrein.

Pepsitocin, the kinin released from human kininogens by pepsin has been purified to homogeneity. Quantitative amino acid analysis gave. 1.1 (1) Ser, 2.7 (3) Pro, 1.4 (1) Gly, 0.7 (1) Met, 2.2 (2) Phe, 1.0 (1) Lys, 1.9 (2) Arg (theory for Met-Lys-bradykinin in parentheses).

Kallikrein has been localized in the distal tubular cells of the kidney cortex by means of fluorescent antibody to rat urinary kallikrein.

PHS-6040
(12-75)

Objectives: Purification of glandular kallikreins and components of the plasma kinin, clotting and fibrinolytic systems for characterization purposes and for production of specific antiserums. Preparation of purified specific antibodies for biochemical and other studies. Preparation of affinity adsorbents from purified antibodies, antigens, enzymes, inhibitor for purification and other purposes, such as devising specific biochemical and radioiummunochemical assays. Applications of these purified proteins, affinity adsorbents, and assay methods to studies of human disease states, such as hypertension.

Major Findings: 1) Human Plasma Kininogens. a) Immunology
Two sheep immunized with B4α, β, and γ in different sequences produced antiserums which contained high-titer antibodies to all kininogens, as well as a relatively low titer antibody only to B4 kininogens. The antibody to kininogens in general is designated Ab_{Kgn}, while the antibody to X, the antigen unique to B4 kininogens, is called Ab_X. Ab_{Kgn} was obtained by slowly passing anti-B4 serum through an agarose column of immobilized kininogens B1 + B2 ("B1,2-agarose") at 5°, washing with several column volumes of PBS. (0.15 M NaCl-0.01 M NaP_i, pH 7.0), eluting with one-fourth to one-third column volume of 8 M guanidinium chloride, pH 4.5, and dialyzing the guanidine eluate against several changes of PBS 6.0. This fraction was called $Ab_{B1,2}$. The Ab_X still remaining in the B1,2-agarose filtrate was obtained apparently pure by immunoaffinity chromatography as above but on a column of B4β + B4γ (overlap region between B4β and B4γ gel filtration peaks) immobilized on agarose ("B4β, γ-agarose").

Since human plasmas entirely deficient in kininogen are now available (Williams and Flaujeac plasmas: see Annual Report for 1975, Serial No. Z01 HL 1944-18 HE), it is possible to critically test kininogen antiserums and purified antibodies by immunoecectrophoresis. With this method, the sheep antiserums to B4 kininogens showed many precipitin arcs with Williams plasma. The $Ab_{B1,2's}$ described above and the antibodies obtained from these antiserums still contained one or more non-kininogen arc. On the other hand, Ab_X gave only a weak, diffuse precipitate (not an arc) just anodal to the origin. This may be nonspecific reaction between sheep Ig and some normal component of human plasma, perhaps also Ig. This precipitate was found only with whole plasma. Ab_X gave precipitin arcs only with A4 and B4 kininogens. Since peak A3 also showed, on retesting, little or no activity in the Hageman factor activation and clotting assays, the +'s for Peak A3 in the table on page 3 of our 1975 annual report should be changed on -'s Thus, the parallelism between the A1-A4 and B1-B4 peaks is greater than previously thought.

Various antiserums and purified antibodies to LMW kininogens I and II (see annual report for 1966) were also tested by immunoelectrophoresis against Williams plasma. All of the antiserums contained variable amounts of minor impurities, as did also all of the AB_{Kgn} preparations obtained from these antiserums by immunoaffinity chromatography, though to a lesser degree than the starting antiserums. The best Ab_{Kgn} preparation was obtained by immunoprecipitation at equivalence of defibrinated human plasma and a sheep antiserum to LMW kininogen I, followed by hydroxyapapite chromatography of a

solution of the washed precipitate in 8 M urea. The antibody, "Ab_{Kgn}", recovered by dialysis of the 8 M urea filtrate against phosphate-buffered saline at pH 6.0, contained no detectable kininogen antigen (see annual report for 1967) and gave no precipitin arcs with Williams plasma in immuno-electrophoresis, but gave, like Ab_X, a diffuse precipitate near the origin.

b) Pepsitocin (Met-Lys-bradykinin). To obtain more peptic peptide from human kininogen for additional identification experiments (see annual report for 1975, Serial No. Z01 HL 1944-18 HE), we wished to use Prep B in order to spare the more valuable B3.2 . However, treatment of Prep B, from which the guanidine had been removed, with 30 times the level of pepsin required for the complete release of kinin in 0.5 h at 37° from a comparable amount of B3.2 gave no detectable kinin. The Prep B employed had been stored in 4.1 M guanidine chloride at 5° and just before use was filtered through a column of Sephadex G-25 at 5° in PBS 6.0 containing 1 mM benzamidine-HCL. The protein fraction had the same kininogen activity with human urinary kallikrein as it had when tested several months previously--i.e., ca 65% of the kininogen activity initially present in the starting Prep A or plasma. However, affinity chromatography of Prep B on columns of Ab_X-agarose gave a filtrate, which contained active kininogens but no X antigen (Ouchterlony) and which readily yielded a kinin when treated with low levels of pepsin. Accordingly, 31 A_{280} units of this kininogen fraction (containing ca 300 mmole of kinin) was treated with 0.5 mg of crystalline porcine pepsin for 20 min at 37° and pH 2.0. The solution was then stirred with 0.25 g of IRC-50 for 1.3 h at 25° and pH 4.0 to adsorb the peptic kinin. The absorbant was successivley eluted with 1 M, 9 M, and 10 M HOAc. The freeze-dried 9 M eluate (13.3 mg dry weight), which contained most of the recovered kinin activity, was dissolved in 0.5 ml of 1.0 M HOAc and filtered through a 1.15 x 121 cm column of Bio-Gel P-4 in 1.0 M H)Ac at 25° and at a rate of 5 ml/h. The kinin activity was found between 78.5 and 83.5 ml of effluent (the rention volumes of authentic samples of BK, LBK, and MLBK on the same column were 86-94, 75-83, and 75-83 ml, respectively). A solution of the freeze-dried, pooled active fraction (0.6$\pm$ 0.1 mg dry weight in 0.5 ml of 0.05 M Tris-HCL-0.08 M NaCl, pH 8.0, was added to a 0.6 x 11.0 cm column of SP-Sephadex C-25 previously equilibrated with the pH 8.0 buffer, and developed at 25° with this buffer at 4 ml/h. Fractions of 2.0 ml were collected and tested for kinin activity on the guinea pig ileum. Activity was found in one peak between 42 and 52 ml of effluent. Using the same column and conditons, the retention volumes of authentic bradykinin, Met.Lys-brady-kinin, and Lys-bradykinin were found to be 12-20 ml, 44-55 ml, and 64-78 ml, respectively. The pepsitocin activity peak was freeze-dried, dissolved in 0.25 ml of 1.0 M HOAc, and gel filtered on the same P-4 column used above, to remove the salts and any other impurities with retention volumes different from that of pepsitocin (e.g., amino acids). Unfortunately, just as the kinin activity was being collected, the fraction collector jammed so that a considerable fraction of the activity was lost. The recovered material now contained 45 µg (35 nmol) when compared on the ileum with authentic Met-Lys-bradykinin, the potency of which had been very accurately determined. Nevertheless, quantitative amino acid analysis (performed by Dr. E. Appella) of 2 nmol of the pepsitocin thus obtained gave

1.1 (1) Ser, 2.7 (3) Pro, 1.4 (1) Gly, 0.7 (1) Met, 2.2 (2) Phe, 1.0 (1) Lys, 1.9 (2) Arg (theoretical values for Met-Lys-bradykinin given in parentheses).

c) HMW kininogens B4β and B4γ as cofactor for surface-mediated activation of Hageman factor. It is now well-documented that HMW kininogen is a normal cofactor for the surface activation of Hageman factor (see Publication 1 below; Wuepper et al., J. Clin. Invest. 56:1663-1672, 1975; and several papers presented at the FASEB meetings on 15 April 1976 and abstracted in Fed. Proc. (FASEB) 35: 692, 1976). Especially critical were the experiments done with highly purified HMW kininogens B4β nd B4γ (described in the 1975 annual report, by M. E. Webster, A. P. Kaplan and R. W. Colman. These kininogens, at 10% of the calculated concentration of HMW kininogen in normal plasma, potentiate 2- to 3-fold the activity of already activated Hageman factor as measured by the activation of its three natural substrates. This enhancement was observed also for low molecular weight Hageman factor fragments in the absence of a surface in their ability to activate plasma prekallikrein. Although the B4 kininogens may be only about 80% pure based on their kinin content, they may be much more pure with respect to the Hageman factor cofactor. The tail of the DEAE-cellulose B4 peak, while containing no kininogen activity, nevertheless is almost as active as B4β or B4γ in cofactor activity.

2) Localization of kallikrein in rat kidney. Dr. Kjell Nustad and colleagues have localized kallikrein in the distal tubular cells of the renal cortex by use of fluorescent antibody to rat urinary kallikrein. The kallikrein and its monospecific antibody were prepared at NIH (see annual report for 1971 and Nustad & Pierce, Biochemistry 13:2312, 1972).

3) Presence of kininogen fragments in human urine concentrates. All normal male urine concentrates so far tested against monospecific AB_{Kgn} gave two precipitin lines, both of which showed identity with the single line obtained with human plasma or with highly purified LMW kininogen B2α. No precipitin arc was found with Ab_{X}. The simplest hypothesis is that a plasma kininogen or a kininogen synthesized by the kidney has been cleaved into two antigenic fragments. Evidence for active kininogen in freshly voided urine has recently been obtained by J. J. Pisano and co-workers.

Significance to Biomedical Research and the Program of the Institute: Highly purified components of the plasma kallikrein-kinin system are crucial to investigations of its physiological function. This system is activated simultaneously with the intrinsic blood coagulation and lysis systems. It now appears that, besides plasma prekallikrein, HMW kininogen also is necessary for the surface activation of Hageman factor which then acts on its three natural substrates, thus initiating the three systems mentioned. It will, therefore, be important to study the chemistry of pure HMW kininogen and the mechanisms whereby it acts.

Proposed Course of Project: We plan to purify the B4 kininogens further to obtain material suitable for chemical and other studies.

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Colman, R. W., Bagdasarian, A., Talamo, R. C., Scott, C. F., Seavey, M., Guimaraes, J. A., Pierce, J. V., and Kaplan, A. P. Williams trait: Human kininogen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with abnormalities of the Hageman factor-dependent pathways. J. Clin. Invest. 56:1650-1662, 1975.

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Webster, M. E. Guimaraes, J. A., Kaplan, A. P., Colman, R. W. and Pierce, J. V. Activation of surface-bound Hageman factor: preeminent role of high molecular weight kininogen and evidence for a few factor. Adv. Exp. Med. Biol., in press.

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

The Section on Experimental Atherosclerosis was elevated to laboratory status this year and renamed the Laboratory of Experimental Atherosclerosis, consisting of the Vascular Physiology Section and the Comparative Atherosclerosis and Arterial Metabolism Section. Both groups continue to work as a unit whose central research mission is targeted on the mechanisms leading to the genesis of the atherosclerotic plaque, the lethal lesion of atherosclerosis. The ultimate aim of this program is to identify each of the steps in the evolutionary sequence of this disease process in terms of cellular metabolic mechanisms and physical chemical laws so that deliberate strategies can be developed to interrupt this sequence at various selected points of vulnerability. This broad objective has required the development of a multidisciplinary program consisting of experimental pathology, human pathology, lipoprotein chemistry, physical chemistry, arterial tissue culture, vascular physiology and data processing. Since the overall program plan has been detailed in previous annual reports, to which the reader may refer for further information, only the salient features of this year's efforts will be reviewed.

Development of Animal Models for Studies of Thrombo-Atherosclerosis

Comparative studies of dietary induced atherosclerosis in dogs, miniature swine, and Patas monkeys have allowed us to define the metabolic parameters necessary to produce arterial disease with characteristics resembling human atherosclerosis. The source of the dietary fat, fed in association with cholesterol, has been linked to the type, distribution, severity, and complications of the atherosclerosis. Cholesterol-rich diets which contain saturated fats, such as beef tallow or hydrogenated coconut oil, cause severe atherosclerosis associated with thrombosis in dogs. Diets which contain unsaturated fats e.g., cottonseed oil, results in much less fulminant disease and no thrombosis. The association of thrombosis and atherosclerosis has also been observed in swine fed a saturated fat diet. Our studies provide for the first time a reproducible model in which to study the role of the hemostatic processes, including platelet physiology, in the initiation and/or progression of the disease process. Changes in platelet membrane fluidity resulting from altered fatty acid composition and cholesterol content is thought to be responsible for the sensitization of platelets to aggregation and thrombus formation. Studies are in progress to define the lipid composition of the membranes of platelets from animals on the various dietary protocols and to attempt to correlate these findings with changes in platelet function. The platelet function studies which are being done in collaboration with Dr. Russell M. Jaffe, Clinical Pathology Department, include the responsiveness of platelets to aggregation in vitro and measurements of platelet survival in vivo. Preliminary studies suggest that platelets from dogs on a saturated fat-cholesterol diet are promptly "sensitized" to aggregation whereas platelets from dogs on an unsaturated fat-cholesterol diet are not.

Disease and Permeability Topography

All animals coming to necropsy are given Evans Blue dye as a visual tag for albumin 24 hours prior to necropsy so that areas along the vascular tree which have an increased permeability to plasma substances will stain blue. The topographic distribution of the blue staining may then be compared to that of the intimal lipid deposition following Sudan IV staining. Thus, areas of lipid deposition are stained red, areas of increased permeability blue, and the normal intimal surface remains white. Generally speaking, the patterns of sudanophila in experimental animals are strikingly similar to the patterns of Evans Blue staining in normal animals. As discussed in last year's annual report this is evidence for the role of increased filtration of plasma substances in the genesis of this disease.

The development of a standardized format for examining the topographic distribution of experimentally induced disease has proved to be a major advance in our methodology. This standardized format permits careful detailed comparisons of the disease process at identical sites from animal to animal. Using this technique in a recently completed study in experimental atherosclerosis in swine, it was possible to demonstrate that the topography of the disease is extraordinarily congruent from animal to animal. This observation represents a major step forward and is of fundamental importance not only in dramatizing the role of localizing factors in this disease but also in the development of new strategies in experimental design to gain a deeper insight into the associated atherogenic processes.

The characteristic topographic pattern appears early in the disease and is characterized initially as simple fatty streaking. However, in a matter of months certain areas in the pattern evolve into typical atheromatous plaques. This is particularly true of the coronary arteries, lower abdominal aorta, and larger peripheral arteries. The demonstrated predicability of this process at given locations permits us now to design new studies to follow the sequence of events leading from the "earliest lesion" on through the complicated lethal atheromatous plaque. This capability was heretofore unavailable for lack of a standardized quantitative approach to the disease topography.

Characterization of the Lipoproteins Associated with Accelerated Atherosclerosis

Detailed characterization of the plasma lipoproteins associated with cholesterol feeding in dogs, miniature swine, rats, rabbits, and Patas monkeys has led to the identification of certain consistent features of the atherogenic hyperlipoproteinemia. These include: 1) the production of a lipoprotein similar to the B-VLDL which occurs in human Type III hyperlipoproteinemia; 2) an increase in the low density lipoproteins and the intermediate lipoproteins which may arise from VLDL catabolism; and 3) the identification of a previously undescribed lipoprotein referred to by us as HDL_c. Associated with all of these lipoproteins is an increased prominence of a specific lipoprotein protein, the "arginine-rich" apoprotein. Characterization of these lipoproteins and their apoproteins in the various species mentioned above is now completed. Studies are in progress to determine the origin and metabolic fate

of these lipoproteins. Particular emphasis is being placed in the role of the "arginine-rich" apoprotein and the HDL_c in the regulation of cholesterol metabolism.

Arterial Metabolism in Tissue Culture

Extension of our *in vivo* studies to *in vitro* tissue culture studies has allowed us to determine that the "arginine-rich" apoprotein and the HDL_c can transfer cholesterol to arterial smooth muscle cells, lead to cholesteryl ester accumulation and regulate cholesterol synthesis by supressing HMG-CoA reductase activity. It is now known that at least two lipoproteins, the LDL and HDL_c associated with cholesterol induced atherosclerosis, can bind to smooth muscle cells and fibroblasts and be taken up by these cells through identical cellular mechanisms. The use of this unique lipoprotein, HDL_c, provides a useful probe to determine the nature of the cell surface receptor and the mechanisms involved in the regulation of cholesterol metabolism.

In addition to arterial smooth muscle cells and fibroblasts, arterial endothelial cells are being grown and maintained in tissue culture. The study of the uptake, transport, and metabolism of plasma components including lipoproteins by endothelial cells in culture provides direct support in our investigations of cellular mechanisms in the intact aorta using the previously described aortic support methodology developed in this laboratory.

Transvascular Transport

Atherosclerosis may be viewed not only as a problem in cellular metabolism but also in most general terms as a problem in transvascular transport of lipid, i.e., lipid is being deposited in the arterial tissue more rapidly than it is being carried away. This concept has been reinforced by our observations (discussed in greater detail in previous annual reports) that the topographic distribution of intimal lipid deposition in most experimentally induced atherosclerosis is very similar to the topographic pattern of increased endothelial surface permeability to plasma substances that is observed in normal animals. For these reasons, considerable effort has been expended in identifying the nature of the macromolecular transport processes that occur in normal arteries. This part of the program has required the development of many new experimental techniques for measurement of diffusion coefficients, permeability coefficients, partition coefficients, binding coefficients, chemical reaction coefficients, tissue-fluid drift velocities, and reflection coefficients. These measurements have been incorporated into a variety of mathematical modeling procedures which have culminated in the development of a set of mathematical expressions which appear to describe the transvascular transport of albumin quite accurately. Although the technical details of the foregoing exceeds the scope of this report it is of interest to note that the transport of macromolecules through the arterial media occurs both by diffusion as well as convective processes and that considerable binding and metabolic degradation can occur along their journey. Two principal barriers to transport exist at or near the vascular interface; one is identified with the endothelial surface and the other with the basement membrane and internal elastica. Numerical values for the permeability coefficients of each of these

structures have been determined. These are the principal barriers to transport in the overall system and appear to play central role in the nature of the disease that will occur at a given site. Hopefully the details of these important relationships between transport parameters and the type of disease process will appear in next years annual report

Concluding Remarks

The multidisciplinary nature of this program and large amount of work that has been done has required broad collaborative and contractural support. We are particularly fortunate to have the enthusiastic collaboration of the Veterinary Resources Branch, DRS, in our swine, canine, and primate colony studies which has made it possible to move all of our colony work from research contracts to "in-house" collaboration. This has resulted in a significant economy both monetarily as well as scientifically. It is hoped that this new area of joint research effort among members of different divisions at the NIH can become a permanent partnership for the common good.

The need for contractural support in the area of laboratory services remains. Four areas of contract service support are absolutely essential to our program since we do not have the necessary space or personnel to carry out these important functions at the NIH: 1) preparative protein/lipoprotein chemistry, 2) tissue culture, 3) iodination of proteins, and 4) ultrastructural analysis (SEM, TEM, and electron probe analysis). Since we will have a continuing need for these services they more appropriately should become "in-house" activities; however, the prospect of acquiring either the space or the positions to achieve this in the reasonable future appears unlikely under the present system of Institute priorities. Although the program necessarily requires that we continue the use of these service contracts, it should be pointed out that the nature of this type of intramural support and the associated price tags are quite visible to the various extramural groups who review them. Certain members of these review bodies have already complained unofficially to us regarding this approach to the support of intramural research.

In view of this, it is likely that our annual defense of each of these contracts will become too tedious for this to remain a practical means of program support. It is hoped that these problems will be recognized and solved before they affect adversely this important area of intramural research.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02801 04 EA

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The arterial intimal tissue responses following endothelial injury.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Fry	Chief, Lab. of Experimental Atherosclerosis	EA NHLI
OTHER:	V. J. Ferrans	Visiting Scientist	OD-P NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.45	.05	.40

SUMMARY OF WORK (200 words or less - underline keywords)

Functional and structural changes in the arterial wall following the endothelial erosion of the surface by increased fluid shear stress.

PHS-6040
(12-75)

Project Description

Objective: To study the functional and structural changes in the arterial wall following the erosion of the endothelial surface by increased fluid shear stress.

Methods Employed: Detailed methodology for this project appears in a previous report. Briefly, the endothelial surface of the descending thoracic aorta in a group of dogs was discretely removed using an inflated arterial balloon. The animals were allowed to recover and were examined at selected times following endothelial cell erosion using both light microscopic and electron microscopic techniques to examine the sequences of events leading to repair of the damaged arterial surface. Concomitant estimates of trans-endothelial macromolecular transport were carried out.

Major Findings: The sequence of repair were as follows: There was immediate generalized platelet adhesion to the eroded surface which was followed in a matter of days by a rapid proliferation of modified smooth muscle cells. Albumin transport into the arterial wall was increased from 10 to 15-fold both in the acutely eroded surface as well as the surface subsequently covered with smooth muscle cell proliferation. At a point in time corresponding roughly with the reappearance of a normal endothelial cell cover, both the proliferative response and the increased trans-intimal transport of albumin decreased.

Significance to Biomedical Research and the Program of the Institute: The atherosclerotic processes are classically characterized by fibromuscular hyperplasia and lipid deposition. The sequence of events leading to this thickened intima are not understood. The topography of intimal proliferative processes in the diseased state frequently corresponds to areas that may have been exposed to increased mechanical stress and/or injury. If the mechanisms of these naturally-occurring processes in this important disease are to be understood, it is essential that we understand in greater detail the factors inducing and controlling the reparative processes occurring at the vascular interface.

Proposed Course of the Project: A large amount of histologic and electron microscopic material has been generated from the above studies and is still in the process of analysis. The major problem in the interpretation of these data is the lack of precision with which the exact extent and area of the initial injury can be defined, particularly in the chronic preparations. It is not possible without this information to establish unequivocally the exact relationship between the reappearance of the normal endothelial surface, the "shutting-off" of the smooth muscle cell proliferative response, and the reappearance of normal macromolecular transport into the vascular interface. This project has been discontinued temporarily while new methodology is being devised which will allow us to create and subsequently identify a well-defined controlled, discrete region of endothelial erosion. The prototype device that is being developed mounts on the end of a vascular catheter, has retractable vanes that can be extended remotely by the operator to sweep off the adjacent

endothelial cells and at the same time inject India ink (biological grade) into the subjacent tissue. The ink "tattoo" particles serve as markers for following the trajectory of the subsequent tissue responses in relation to the precise site and extent of injury.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02802 04 EA

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The relationship of arterial intimal Evans blue dye concentration to surface reflectance and light absorption.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald L. Fry Chief, Lab of Experimental Atherosclerosis EA NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	.30	1.20

SUMMARY OF WORK (200 words or less - underline keywords)

To establish and validate the relationship between the concentration of Evans blue dye in the vascular interface and the absorption of red light.

422

PHS-6040
(12-75)

Project Description

Objective: To establish and validate the relationship between the concentration of Evans blue dye in the vascular interface and the absorption of red light.

Methods Employed: A special reflectance measuring system was designed and constructed, such that opened vessel surfaces could be viewed under uniform illumination through a lens system on a large glass screen. A small aperture in the center of this screen led to the sensing surface of a photomultiplier tube. Appropriate light filters could be inserted in this light path as needed. The opened vessel surfaces were held in a specially designed servo-motor-driven tray submerged in saline, both to act as a metabolic supporting milieu for the vessel surface, as well as providing a nonreflecting optical coupling to the photo-sensing system. Various regions of the vessel surface could then be maneuvered by the operator under visual control such that a very small region on the vessel surface could be placed under the aperture in the screen. With a Wratten 29 filter in the light path it was possible to measure the amount of red light that is absorbed and reflected from the vessel surface. By staining various regions on the vessel surface to varying depths it was possible to establish a monotonic relationship between the optical absorbance ρ (negative log of the ratio of light intensity from stained surface to that from adjacent unstained surface) of the stained surface and time (duration of exposure to EBD-albumin complex). A reflectance model based on the Lambert-Beer law of light absorption was evaluated to represent this relationship by fitting model parameters to data obtained as dye was eluted back out of the surface using an untagged albumin solution. The final model that was chosen implicitly predicts the optical absorption coefficient for EBD from these fitted parameters and also the change in vessel surface area that occurs with stretch of the wall. The accuracies of these implications were tested by measuring the EBD absorption coefficient directly in an Aminco microphotometer and by measuring the correlation between measured change in wall stretch with those predicted from reflectance measurements.

Major Findings: It was found that the accumulation of EBD in the vascular intima bears an approximately logarithmic relationship to the amount of light absorbed as given by $M = k_1\rho + k_3\rho^3$, where M is the surface accumulation in n·moles/cm^2 and ρ is the optical absorbance. The constants k_1 and k_2 were found to be 3.92 and 0.80, respectively.

The validity of the model was established by noting that the predicted value of the absorption coefficient (from the fitted parameters) was 0.128 as compared to 0.126 for the directly measured value. The linear regression of the predicted wall stretch data on directly measured values had a slope of 0.99, a negligible intercept, and a correlation coefficient of 0.99. We conclude that the model represents the actual physical state with good precision.

Significance to Biomedical Research and the Program of the Institute: When Evans blue dye is injected intravenously into an animal virtually all of the dye becomes bound to serum albumin. Knowing the molar ratio of Evans blue dye to albumin permits the use of Evans blue dye as a measurable visual tag for albumin. Measurement of the amount of Evans blue dye in the intima thus can be used as a measure of transvascular macromolecular transport. We have shown that the atherosclerotic process is greatly enhanced in regions of increased EBD accumulation (increased transvascular transport of plasma proteins). The ability to quantify the topography of permeability in the opened vascular interface with this relatively simple reflectometric scanning technique opens many new areas in the study of the mechanisms of atherogenesis.

Proposed Course of the Project: This methodology is being applied first to studies designed to quantify the correlation between the topography of vascular permeability and the topography of the atherosclerotic lesions in animals. The second major area of application will be to quantify the permeability changes that occur with various altered hemodynamic events known also to be related to an increased atherosclerotic process. The manuscript describing this technique in detail as well as the physical chemical interpretation of Evans blue dye accumulation has been submitted to the American Journal of Physiology for publication. This aspect of the project now has been terminated.

Publications

Fry, D. L. Hemodynamic forces in atherogenesis. In: Cerebrovascular Diseases, Tenth Princeton Conference, P. Steinberg, M.D., editor. New York: Raven Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02803 03 EA

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The study of arterial transport processes in an in vitro life support system.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Fry	Chief, Lab. of Experimental Atherosclerosis	EA NHLI
OTHER:	R. W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLI
	R. J. Lutz	Chemical Engineer	BEIB DRS

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, Division of Research Services

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	.20	1.00

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this research was to develop an in vitro arterial organ support system for study of transvascular transport mechanics and short range metabolic mechanisms.

PHS-6040
(12-75)

Project Description

Objective: To develop an in vitro arterial organ support system for study of transvascular transport mechanics and short range metabolic mechanisms.

Methods Employed: A device has been developed which consists of a leaded glass chamber, the environment of which is thermostatically and humidistatically controlled by using appropriate electronic feedback systems. A carefully removed arterial segment is placed in a specially designed holding device which permits control of the mechanical constraints on the blood vessel as well as anchoring the vessel for application of a "well assembly" and "pressure manifolding system" in the above chamber to permit selected areas of the opened vascular interface to be studied under controlled, chemical, thermal, and mechanical conditions.

Major Findings: Tissues studied in the above system have been found to remain viable by both physiological and ultrastructural criteria at least two hours. The tissue should be viable for periods in excess of 24 hours with provision for profusion of the adventitial surface of the vessel and refreshment of the milieu in contact with the endothelial surface. The macromolecular conductance of the vascular interface for albumin has been found to be greatly increased by subtle mechanical injury, temperatures in excess of 40°C, stretching the surface in excess of 200%, exposing the surface to saline, exposure to certain foreign proteins, brief exposure to air, and exposure to various simple polar solvents such as ethanol. Moderate increases in conductance were produced by concentrations of epinephrine, noradrenalin, and histamine in levels of approximately one order of magnitude above "physiologic" serum levels. Serotonin, adenosine diphosphate do not appear to alter the wall conductance.

Significance to Biomedical Research and the Program of the Institute: This is an in vitro technique that has been developed in which the transendothelial, intimal and medial transport processes of arterial tissue can be studied under controlled mechanical, thermal and chemical conditions for periods of at least two hours and probably considerably longer with continuous refreshment of the milieu and purging of the adventitial surface. This degree of experimental control is not possible in the in vivo situation and, therefore, this new methodology opens many new avenues for studying transport mechanics otherwise not available. Evidence described elsewhere from this laboratory strongly suggests that increased interfacial transport of macromolecules is one of the central factors in the atherogenic process. Consequently, this new methodology should lead to important new insights in this disease process.

Proposed Course of the Project: This phase of the project has been finished and a manuscript is in preparation. Many aspects of this work will appear as part of various other publications including the one noted below. This methodology has now become a standard technique for in vitro study of transport phenomenon in this Laboratory.

Publications

Fry, D. L. Hemodynamic forces in atherogenesis. In: Cerebrovascular Diseases, Tenth Princeton Conference, P. Steinberg, M.D., editor. New York: Raven Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02804 02 EA

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The arterial ultrastructure in areas of increased and decreased transvascular transport.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. L. Fry Chief, Lab. of Experimental Atherosclerosis EA NHLI

COOPERATING UNITS (if any)

Tousimis Research Corporation under contract #NIH 75-C-256-CC

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.10	.10	2.00

SUMMARY OF WORK (200 words or less - underline keywords)

To establish the ultrastructural characteristics of the endothelial surface and subendothelial tissues in areas of increased and decreased transendothelial transport of Evans Blue Dye tagged albumin.

PHS-6040
(12-75)

Project Description

Objective: To establish the ultrastructural characteristics of the endothelial surface and subendothelial tissues in areas of increased and decreased transendothelial transport of Evans blue dye tagged albumin.

Methods Employed: The topographic distribution of increased transendothelial albumin flux was determined using the Evans blue tagging technique in four, two-year old foxhounds and in two, one-year old minipigs by administering the dye twenty-four hours prior to study. At the time of study each animal was anesthetized with pentobarbital, and both the axillary arteries and inferior vena cava were cannulated for pressure profusion with buffered saline and fixation with three percent buffered glutaraldehyde for one hour. The arterial trees were then carefully dissected free and sets of specimens were removed for transmission and scanning electron microscopy from areas of increased transendothelial albumin transport and from areas of normal transport.

Major Findings: By scanning electron microscopy, the tissues from normal transport areas showed a highly ordered endothelial surface pattern with distinct cell borders while tissues from high transport areas showed a high degree of disorder and pleomorphism with poor definition of cell borders. Transmission electron microscopy showed normal endothelial architecture in both types of regions but differences in the subendothelial space. Normal regions demonstrated a more ordered and densely packed pattern of intimal connective tissue (collagen, elastic fibers, and elastin) whereas tissues from high transport areas showed a much looser connective tissue pattern with many open spaces characteristic of moderate interstitial edema.

Significance to Biomedical Research and the Program of the Institute: The localized development of the atherosclerotic process has been shown to correspond to regions of antecedent increased transendothelial transport of albumin and perhaps of other macromolecules such as lipoproteins. The above observations suggest that this disease process may result from increased flux of atherogenic precursors across regions of structurally and functionally abnormal endothelial cells.

Proposed Course of the Project: Manuscript in preparation. This study is discontinued.

Publications

Fry, D. L. Hemodynamic forces in atherogenesis. In: Cerebrovascular Diseases, Tenth Princeton Conference, P. Steinberg, M.D., editor. New York: Raven Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02805 02 EA

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The mechanics of albumin transport into the arterial intimal medial space.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLI
OTHER:	R. W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLI
	K. H. Weisgraber	Senior Staff Fellow	EA NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	.75	2.00

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to establish further a valid physical chemical model for macromolecular transport into the arterial wall.

PHS-6040
(12-75)

Project Description

Objective: To establish further a valid physical chemical model for macromolecular transport into the arterial wall.

Methods Employed: A specially designed in vitro test system described elsewhere was used to measure the flux of ^{125}Iodine tagged albumin and Evans Blue dye (EBD) tagged albumin into the arterial wall. This system permitted the arterial surface to be exposed to the tagged albumin under controlled conditions for various selected periods of time. Special experimental maneuvers were devised such that it was possible to study the uptake of albumin, as a function of duration of exposure to the tagged albumin, location along the arterial segment, degree of wall stretch, and transmural pressure. Uptake was measured under these conditions for the normal endothelial surface, the lightly injured surface, and the heavily injured surface. These two levels of controlled injury were devised to assist in locating the various barriers to transport. The lightly injured surface was shown be scanning electron microscopy as well as transmission electron microscopy to have an absent endothelial cell layer but an intact basement membrane and internal elastica. The heavily injured surface demonstrated not only loss of the endothelial surface but also disruption of the basement membrane and internal elastica.

Major Findings: Evidence was presented in last year's report showing that the albumin transport into the heavily injured wall can be explained by simple diffusion into a homogeneous slab (the wall) from an adjacent liquid phase (the blood) using a simple solution to Ficks second law of diffusion. By changing the boundary conditions for this solution to include a diffusion barrier at the interface of these phases it was possible to show that simple diffusive kinetics can also explain the transport of macromolecules in the lightly injured case (no endothelial cells but intact basement membrane and internal elastica).

In the arterial system, a second driving force in addition to the above exists, i.e., the pressure acting across the wall. Pressure not only provides a second driving force but also causes increased wall stretch thereby potentially altering the coefficient of diffusion but, more particularly, alters the permeability coefficient for the interfacial barriers (the endothelial surface, basement membrane, and internal elastic lamella). The transport mechanics under these conditions was studied by measuring the uptake of tagged albumin as a function of imposed transmural pressure differences, both in the positive and negative directions. Comparison of the uptake curve for the positive transmural pressure to that for the negative transmural pressure allowed calculation of the increment increase influx into the wall related to the pressure alone and also to the imposed degree of stretch alone. The pressure driven flux into the wall was found (at physiologic levels of pressure) to be of the same order as that for the steady state diffusive flux into the wall. Thus, both driving forces must be taken into account in considering transport mechanics into the arterial wall and, under conditions of hypertension, it is likely that the pressure driven or convective flux may become the dominant factor.

Significance to Biomedical Research and the Program of the Institute: A large body of evidence both from this laboratory as well as from others indicate that the atherogenic precursors probably come from the blood phase into the wall as various complexes with proteins. There is also some evidence that dissociation in the intimal space may occur with preferential binding of lipid substances in the tissue matrix. While albumin itself can carry both fatty acids and some cholesterol, its major interest to us is related to its use as a prototype model for macromolecular transport and binding in the intimal space. Thus, while of inherent interest in albumin and fatty acid transport, the above studies begin for the first time to place macromolecular transport in vascular tissue on firm physical-chemical grounds and allow us to sharpen our technical, physical, and mathematical tools which will be essential in subsequent studies of the transport processes for the atherogenic precursors (as these become better defined). As these transport mechanisms come into clear perspective it should be possible to gain deeper insights into the transport processes associated with the development of fatty streaking at certain sites along the aorta, the curious propensity of certain sites to go on and develop the lethal atheromatous plaque, and to gain a deeper insight into the puzzling tri-color lesion of experimental atherosclerosis which is discussed in greater detail elsewhere.

Proposed Course of the Project: The above studies will be expanded to include arteries other than the aorta, to define more explicitly the factors influencing arterial transport processes and to establish more firmly the influence of other physical parameters such as temperature, pH, etc. Data processing and manuscript preparation are underway. An initial manuscript has been submitted to the American Journal of Physiology.

Publications

Fry, D. L. Hemodynamic forces in atherogenesis. In: Cerebrovascular Diseases, Tenth Princeton Conference, P. Steinberg, M.D., editor. New York: Raven Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02806 03 EA

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Blood velocity profiles and hemodynamic stresses in the aorta and its major branches.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. J. Patel	Medical Officer	EA NHLI
OTHER:	H. B. Atabek	Professor, Catholic Univ. of America	
	S. C. Ling	Professor, Catholic Univ. of America	

COOPERATING UNITS (if any)

Mechanical Engineering Department and Department of Civil Engineering, Catholic University of America, Washington, D. C.

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology Section

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.4	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study the blood velocity profiles and hemodynamic stresses in the aorta and its major branches. The topics of present interest are: 1) Measurement of local flow fields in the coronary artery of dogs; and 2) experimental validation of the nonlinear theory of pulse propagation to predict flow fields at more distal sites along the aorta from pressure-flow measurements made at a proximal site.

PHS-6040
(12-75)

Project Description

Objective: 1) to measure, in vivo, local blood flow fields in the aorta and its major branches, e.g., the left circumflex coronary artery; 2) to test experimentally the nonlinear theory of pulse propagation for predicting flow fields at more distal sites along the aorta using pressure-flow measurements made proximal to the test site; and 3) to use the above theory to predict flow fields near branch sites, e.g., the trifurcation of the aorta in dogs.

Methods Employed: 1) Pressure, pressure gradient, pressure-radius relationships and arterial taper were measured on the left circumflex coronary artery of anesthetized and open-chested dogs over a wide range of blood pressures and flow rates produced by intravenous infusion of Persantine. The velocity profile, wall shear and flow were computed from these data using a nonlinear theory of blood flow. Flow was also measured with an electromagnetic flow meter to provide an independent check. 2) To determine the geometry and the pressure-area relationship in the aortic trifurcation area, a 16 mm cine camera equipped with a high-power telephoto lens was employed. Motion pictures of the pulsating arteries were taken. The arterial pressure was also monitored. The resulting information was used for determining the pressure-area relation. At the end of the filming the animal was sacrificed and the region of interest was filled with a casting material under the mean value of the in vivo pressure. The hardened casts were taken out of the animal and sliced at regular intervals. Enlarged projections of these slices were used to determine the variations of shape and cross-sectional area along the aorta, at the junction and along the branches. 3) The nonlinear theory of pulse propagation developed by Dr. Atabek was tested experimentally in a distensible tapering silicon rubber model. Using the pressure gradient as input at the proximal end, the flow field at the distal end was computed and verified using measured values of pressure and center-line velocity.

Major Findings: The coronary artery study is completed and published. The nonlinear theory of pulse propagation predicted the distal flow fields accurately in a distensible silicon rubber tube. This approach will be tested next in vivo in the dog aorta.

The study of the trifurcation area is still in progress. The geometry and the pressure-radius relationship of this region have been experimentally determined. The pressure-flow relationships are currently under study.

Significance to Biomedical Research and the Program of the Institute: The role of hemodynamic stresses in the etiology of early atherosclerosis is well established by Fry. In order to study this role quantitatively we need to measure, in vivo, flow fields in the critical areas of the circulatory system. The present study of the left circumflex coronary artery and the aortic trifurcation area signifies a modest step toward this goal.

Publications

Atabek, H. B., Ling, S. C., and Patel, D. J.: Analysis of Coronary Flow Fields in Thoracotomized Dogs. Circ. Res. 37:752-761, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02807 03 EA

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Vascular mechanics: arterial wall properties.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dali J. Patel Medical Officer EA NHLI
OTHER: R. N. Vaishnav Professor Catholic University of America

COOPERATING UNITS (if any)

Department of Civil Engineering, The Catholic University of America, Washington, D. C.

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology Section

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project was to determine the local and overall viscoelastic properties of the blood vessel wall under physiologic conditions. The local properties were studied in two ways. The compliance of the intimal layer was studied by exposing the surface to a microindentor (metal rod with a prodder tip) and measuring the indentation of the intimal surface as a function of applied force. The strength of the endothelium was studied by exposing the endothelial surface to a saline jet of known strenth and quantifying the resulting damage to the endothelial cells. These studies will shed added light on the role of mechanical factors in the pathogenesis of atherosclerosis.

436

PHS-6040
(12-75)

Project Description

Objective: To study local and overall viscoelastic properties of the blood vessel wall under physiologic conditions.

Methods Employed: Work was continued on the development of two different methods to study the local rheology of the vascular intima and the endothelial surface. The first, the microindentation method, was designed to study the local compliance of the vascular intima. In this method, a segment of canine middle descending thoracic aorta (MDTA) was excised, slit open longitundinally and stretched to in vivo dimensions in a stretch rack. Small forces (typically 120 mg) were then applied to various locations on the intimal surface using a modified analytical balance, one pan of which was replaced by a metal rod with a prodder tip of 0.19 mm diameter. The displacement due to indentation caused by the tip was measured as a function of time using a differential transformer transducer. The indentation at the end of 30 seconds was used as a measure of local compliance of the vascular intima and its substructure. Various sites on many segments, including sites near regions of intercostal orifices, were studied using this method. During the report period work continued on refinement of the microindentation apparatus, so as to make the device more rugged, stable, and capable of handling complicated geometries in the regions of orifices.

The second method was designed to study the strength of the endothelium using saline jets. An instrument was designed to apply jets at specific sites on the endothelium by modifying a microscope with turret-mounted objectives, one of which was replaced by a jet nozzle (diameter 0.4 mm). A carefully handled aortic segment was stretched as above in a stretch rack and backed by a piece of lucite. Controlled jets of saline were then applied to the endothelium for various durations and resulting lesions made visible by subsequently staining the tissue with Evans Blue Dye. A constant-temperature housing was designed to house the jet apparatus and the specimen so that experiments could be conducted at specified temperatures. A magnified (X 10) lucite model of the jet apparatus was designed to determine shear stress field as a function of radial distance in the model which could then be translated in terms of the prototype values using similarity principles.

Two studies on overall properties of the blood vessel wall were concluded during the report period. The first study involved determination of anisotropic, nonlinear viscoelastic properties of tissue using segments of the MDTA and other blood vessels. The second study involved determination of the elastic properties of canine jugular and human saphenous veins, some of which had been transplanted in the arterial circulation for varying periods prior to study.

Major Findings: The findings on the local compliance of the vascular intima have been published (J Appl Physiol 38:344-350, 1975). Additional work on this project consisted mainly of apparatus modifications. A large number of vessels were studied using the jet method. These studies indicate that it is possible to quantify locally the yield strength of the endothelium. A frequently obtained annular lesion clearly demonstrates that the endothelium

is relatively resistant to damage by normal stresses but rather susceptible to damage by shearing stresses. This corrobrates previous findings of Dr. Fry. The size of the lesion was influenced by jet intensity as well as the duration of jet impingement.

Significance to Biomedical Research and the Program of the Institute: Recent studies by Fry indicate that hemodynamic and local tissue factors play a significant role in the pathogenesis of atherosclerosis. Hemodynamic shear stresses, for example, can alter the permeability of the endothelium to macromolecules in the blood. The jet studies will provide necessary information regarding the strength of the endothelial surface and its permeability to macromolecules. Likewise, it has been observed that regions of the vascular intima sugjected to chronic high unidirectional stresses appear to develop densely oriented collagen in the subendothelial region which apparently protects the intima from subsequent lipid deposition. The studies on local compliance of the vascular intima will provide a rheological counterpart to the histological finding stated above. The work on overall viscoelastic properties is the most general characterization of the mechanical properties of arteries available to date and should help in studying the effects of aging, disease process and pharmacologic agents on tissue properties.

Publications

1. Wesly, R. L. R., Vaishnav, R. N., Fuchs, J. C. A., Patel D. J., Greenfield, J. C., Jr.: Static Linear and Nonlinear Elastic Properties of Normal and Arterialized Venous Tissue in Dog and Man. Circ. Res. 37:509-520, 1975.

2. Plowman, F., Young, J. T., and Janicki, J. S.: An Instrument for Dynamic Measurement of Longitudinal Stresses and Strains in a Blood Vessel In Vivo. Bioreheology 12:21-25, 1975.

3. Vaishnav, R. N., Atabek, H. B., and Patel, D. J.: Some Recent Studies on the Mechanical Properties of the Intimal Layer and Adjacent Flow Fields. Journal of Engineering Mechanics, Division of American Society of Civil Engineers, 1976, in press.

4. Vaishnav, R. N., Young, J. T., Patel, D. J.: Nonlinear Viscoelastic Theory for Large Blood Vessels. Proceedings of the International Congress on System Dynamics, Valley Forge, PA, 1975, in press.

5. Patel, D. J. and Vaishnav R. N.: Mechanical Properties of Arteries. Proceedings of the NATO-Advance Study Institute in Cardiovascular Fluid Dynamics, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02808 03 EA

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Trial of psychophysiologic techniques for the amelioration of hypertension,

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bernard L. Frankel	Staff Investigator	EA NHLI
	Dali J. Patel	Medical Officer	EA NHLI
	David Horwitz	Senior Investigator	HE NHLI
OTHER:	William T. Friedewald	Chief, Clin. Trials Br.	CT NHLI
	E. Freis	Senior Investigator, V.A. Hospital	

COOPERATING UNITS (if any)

Veterans Administration Hospital

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

Reduction of blood pressure through a number of psychophysiologic techniques has been reported. These have included biofeedback techniques, autogenic exercises, hypnosis, and transcendental meditation. Such studies, although demonstrating that blood pressure can be altered, did not provide definitive information on how long the effects were sustained at useful levels and usually were not adequately designed. The present study is intended to determine whether a combination of psychophysiologic techniques including biofeedback and autogenic exercises can produce a substained, therapeutically useful reduction in blood pressure in patients with essential hypertension. Preliminary results suggest that such a combination is not more clinically effective than a placebo procedure; both of these approaches, however, appear to be more therapeutic than no treatment.

PHS-6040
(12-75)

Project Description

Objective: Studies reporting that elevated blood pressure (BP) can be lowered by psychophysiologic techniques such as biofeedback, autogenic exercises, hypnosis, and transcendental meditation have not provided definitive information on how long the effects were sustained at clinically meaningful lower levels and usually were not adequately controlled. The present study was designed to determine whether a combination of psychophysiologic techniques could produce a sustained, therapeutically useful reduction in BP in patients with essential hypertension.

Methods Employed: The study group consists of 22 patients with uncomplicated essential hypertension who show mean diastolic BP levels between 90 and 105 while supine. Patients undergo an initial eight-week period of evaluation during which BP is taken at least once a week by a nurse-observer blind to the patient's experimental status throughout the study. The mean value of the BP measurements of the final 6 of these 8 weeks is used in assessing pre-treatment ("baseline") BP levels. Thereafter, patients enter a sixteen-week study period during which the weekly BP determinations continue. Means of BP data recorded during the final 6 weeks of this 16-week period are compared to the means of baseline measures to evaluate overall therapeutic effects.

First Phase Studies

Patients are randomly allocated to one of three groups:

1. Active Treatment (AT) Group (N=7) - In 20 laboratory sessions, AT patients are trained in the use of electromyographic and/or skin temperature biofeedback and diastolic BP feedback. They also learn autogenic exercises and relaxation training. During laboratory training sessions, pulse, BP, finger temperature, and frontalis muscle activity are monitored noninvasively. These patients also pursue a monitored daily home practice program to reinforce and generalize laboratory learning.

2. Pseudo-Treatment (PT) Group (N=7) - In each of 20 laboratory sessions these patients receive only noncontingent or pseudodiastolic BP feedback arranged to convey a sense of success to them. While laboratory instrumentation and physiologic monitoring is the same for this group as for the AT group, they do not practice any techniques at home.

3. No-Treatment Control (NTC) Group (N=8) - After completing the 8-week baseline period, NTC patients participate in a minimal program consisting of weekly BP determinations by the nurse-observer for 16 more weeks.

Second Phase Studies

PT and NTC patients are offered the opportunity to participate in the AT protocol after completing their initial sixteen-week period, each patient serving as his own control. Patients in any of the three groups having a

decrease in mean diastolic BP to either less than 90 mm Hg or to a level at least 10% below baseline values are studied for an additional two months to determine if this fall is sustained.

Associated Observations

Prospective observations of psychological and hemodynamic status are performed initially. Patients undergo a clinical interview, standard psychological tests, and determinations of cardiac output utilizing a CO_2-rebreathing technique. Findings will be correlated with clinical success in an attempt to delineate the characteristics predictive of success.

Major Findings: Studies of 20 patients have been completed while those of the 2 others are near completion; 7 were in the active treatment group (AT), 7 in the pseudo-treatment group (PT), and 8 in the no-treatment control group (NTC). Of the 7 AT patients,two have shown clinically unequivocal decreases in post-AT supine diastolic BP (from 95.8 to 87.8 mm Hg in one; from 90.5 to 86.5 mm Hg in the other). One AT patient had a modest decrease to a level which was neither less than 90 mm Hg nor more than 10% less than baseline (from 93.7 to 90.5 mm Hg). The other 4 AT patients were treatment failures. Of the 7 PT patients, one showed a modest decrease in the mean post-PT diastolic BP (from 104.3 to 100.0 mm Hg); he, thereafter, participated in active treatment for 16 more weeks and had a further comparable decrease (from 100 to 95.8 mm Hg). Of the 4 PT patients having no post-PT changes in BP, 3 continued with the AT protocol; of these 3, two had no post-AT changes in BP, while one had a clinically meaningful BP fall (from 94.7 to 86.7 mm Hg). Because each of the two other PT patients showed post-PT decreases in diastolic BP to levels below 90 mm Hg (from 90.3 to 86.7 mm Hg, and from 91.5 to 87.2 mm Hg respectively), they did not continue with AT. Seven of the eight NTC patients showed no BP decreases while in one it fell from 97.0 to 93.0 mm Hg. This patient's BP decreased further from 93.0 to 90.0 mm Hg after completing her AT participation. One NTC patient showed no post-AT changes in BP, while two others have not yet completed their AT periods. Four NTC patients chose not to continue on in the AT phase.

While there do not appear to be any clear-cut psychological or physiological patterns of predictive value in those patients who responded to active or pseudo-treatment, detailed statistical analyses are being done to look for less obvious correlations and statistical significance. Follow-up evaluations of those patients who responded to AT are also being carried out to assess whether post-AT decreases in BP have been sustained. The data-collection and patient-training aspects of this study are to be completed by June, 1976.

Significance to Biomedical Research and the Program of the Institute: These findings suggest that the use of a combination of elaborate psychophysiologic techniques in essential hypertension is not clearly more clinically effective than a placebo procedure. However, since both techniques appear to be more effective than no treatment, certain factors common to both

(e.g., regular periods of prolonged relaxation) may be of significant clinical value to some patients with essential hypertension. Future studies might focus more on delineating these patient characteristics predictive of clinical responsiveness.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02809 02 EA

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Quantitation of the apolipoproteins in plasma by two-dimensional immuno-electrophoresis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLI
OTHER:	K. S. Holcombe	Chemist	EA NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.25	.50

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to develop a relatively simple and rapid quantitative immunochemical method for apolipoprotein analysis. The method has been validated using lipoproteins from control and cholesterol fed rats for the arginine-rich and A-I apoproteins. It is presently being extended to the analysis of various swine and human apoproteins.

PHS-6040
(12-75)

Project Description

Objectives: To determine changes in the distribution of apolipoproteins in the plasma of control and cholesterol fed animals and man. Also to monitor changes in cholesterol induced hyperlipoproteinemia following hypolipidemic drug therapy.

Methods Employed: Monospecific antisera has been prepared to the "arginine-rich," A-1, and B-apoproteins of the rat, dog, miniature swine, and human lipoproteins. Laurell's two-dimensional quantitative immunoelectrophoretic procedures as revised by Versey and Davis has been modified for lipoprotein apoprotein quantitation. Our modification of the method, which includes delipidation of samples by triton, has overcome a major difficulty of apoprotein quantitation, i.e., failure to analyze hypertriglyceridemic plasma because of the presence of large, poorly migrating lipoproteins. In addition to allowing quantitation of all plasma samples, delipidation also reduced the possibility of masked antigenicity.

The rat plasma lipoproteins are the principal lipoproteins under investigation at this time. The plasma lipoproteins of rats are fractionated by ultracentrifugation into four major density classes - d<1.006 (VLDL); 1.006-1.019 (intermediate); 1.019-1.063 (LDL and HDL_1 or HDL_c) and 1.063-1.21 (HDL_2). The quantitative distribution of the various apoproteins is determined in each ultracentrifugal fraction for control chow fed animals vs. rats on various hypercholesterolemic diets. The hypercholesterolemic diets contain lard and cholesterol plus a bile acid (either cholic acid or taurocholate). In addition, some of the animals also receive propylthiouracil (PTU). The alterations in lipoprotein metabolism following administration of hypolipidemic drugs is determined by analysis of changes in the apolipoprotein distribution.

Major Findings: Using the two-dimensional quantitative electrophoretic procedure as modified, it has been determined that cholesterol feeding in association with taurocholate and PTU results in a five-fold elevation of the "arginine-rich" apoprotein in plasma. The increase in the "arginine-rich" apoprotein is associated with the occurrence of the beta-VLDL in the d<1.006 fraction. Administration of an experimental drug supplied by the Upjohn Company (U-41,792) results in a reduction of the "arginine-rich" apoprotein in the d<1.006 fraction and an increase in the HDL_c lipoprotein.

Significance to Biomedical Research and the Program of the Institute: A relatively simple and rapid quantitative method for apolipoprotein analysis provides a useful tool to monitor changes in plasma lipoproteins induced by dietary manipulations and drug therapy. These studies will provide insight into changes in lipoprotein metabolism associated with the development of atherosclerosis following cholesterol feeding or the reversal of atherosclerosis following drug therapy.

Proposed Course of the Project: The project will continue along the lines indicated above. The methods will be extended to the analysis of human lipoprotein changes induced by diet and drug therapy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02810 05 EA

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Hyperlipoproteinemia and atherosclerosis: changes in plasma lipoproteins and apolipoproteins induced by cholesterol feeding.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLI
	K. H. Weisgraber	Senior Staff Fellow	EA NHLI
	D. L. Fry	Chief, Lab. of Experimental Atherosclerosis	EA NHLI
OTHER:	K. S. Holcombe	Chemist	EA NHLI

COOPERATING UNITS (if any)
Meloy Laboratories, Springfield, Virginia - NHLI Contract #N01-HI-3-2926.

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	1.00	1.25

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this study to correlate the changes in plasma lipoproteins in animals fed cholesterol-rich diets with the development of accelerated atherosclerosis. The characterization of the lipoproteins induced by cholesterol feeding in dogs, swine, monkeys, rats, and rabbits has established that various species have a similar metabolic response to increased dietary cholesterol. The hyperlipoproteinemia has certain consistent features which include the occurrence of beta-very low density lipoproteins, an increase in low density lipoproteins, and the appearance of a unique lipoprotein, the HDL_C. The occurrence in increased concentration of a specific apoprotein (arginine-rich apoprotein) with all these cholesterol induced lipoproteins suggests an important role for this protein in cholesterol metabolism and possibly in accelerated heart disease.

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PHS-6040
(12-75)

Project Description

Objectives: 1) To characterize the lipoproteins and apoproteins from control dogs, miniature swine, Patas monkeys, rats, and rabbits, and to compare these to changes induced by cholesterol feeding. 2) To correlate the type of hyperlipoproteinemia with the type, distribution and degree of atherosclerosis.

Methods Employed: The various animal species including dogs, swine, monkeys, rats and rabbits were fed diets which contain 0.5 to 2.0% cholesterol as described previously. Isolation of the plasma lipoproteins was accomplished by the combination of ultracentrifugation and Geon-Pevikon block electrophoresis. The purified lipoproteins were characterized with respect to electrophoretic mobility, immunochemical reactivity, size by electron microscopy, chemical composition and apoproteins. The apoproteins were isolated and purified by Sephadex and DEAE column chromatography. Analyses of the apoproteins included amino acid analysis, N- and C-terminal amino acids, and molecular weights.

Major Findings: Dogs, miniature swine, rats, rabbits and Patas monkeys fed high cholesterol diets had a similar lipoprotein response which was associated with the development of atherosclerosis. Animals on a low cholesterol diet served as controls. The characteristics of the hyperlipoproteinemia associated with atherosclerosis were as follows: 1) The beta-VLDL become prominent lipoproteins. The B-VLDL are beta-migrating lipoproteins in the d<1.006 fraction which resemble the beta-VLDL of human type III hyperlipoproteinemia particularly with respect to the prominence of the "arginine-rich" apoprotein. 2) LDL and the intermediate lipoproteins (IDL) are present in increased concentrations and are variably enriched in the "arginine-rich" apoprotein. These lipoproteins and the beta-VLDL may represent remnants of intestinal lipoproteins induced to transport the dietary lipid. 3) A unique class of lipoproteins, which we have called HDL_c, are a consistent feature following cholesterol feeding. These lipoproteins are cholesterol-rich and contain the "arginine-rich" apoprotein and A-1. They lack the B-apoprotein. The HDL_c are important regulators of sterol synthesis in aortic smooth muscle cells and fibroblasts. (See project report entitled, "Tissue culture studies of aortic smooth muscle cells and skin fibroblasts: cell growth and metabolism in response to incubation with various lipoprotein classes.")

Characterization of the "arginine-rich" apoprotein suggest homology of this protein among the species. They have a similar amino acid analysis and contain 12 moles% arginine. The "arginine-rich" apoprotein appears to play an essential role in cholesterol transport between lipoproteins and possibly between lipoproteinsand the aortic wall (atherosclerotic lesion).

Significance to Biomedical Research and the Program of the Institute: Characterization of cholesterol induced hyperlipoproteinemias and development of animal models resembling human disease will enable us to better understand human lipoprotein metabolism. In addition, these studies are designed to correlate the type of hyperlipoproteinemia with the type,

distribution and degree of experimentally induced atherosclerosis.

Proposed Course of the Project: The project will continue along the lines indicated above with an extension in the detailed characterization of human lipoproteins.

Publications

1. Mahley, R. W., Weisgraber, K. H., Innerarity, T., Brewer, H. B., Jr. and Assmann, G.: Swine lipoproteins and atherosclerosis. Changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. Biochemistry 14:2817-2823, 1975.

2. Mahley, R. W., Weisgraber, K. H., Innerarity, T. and Brewer, H. B., Jr.: Characterization of the plasma lipoproteins and apoproteins of the Erythrocebus Patas monkey. Biochemistry, in press.

3. Mahley, R. W., Weisgraber, K. H. and Innerarity T.: Atherogenic hyperlipoproteinemia induced by cholesterol feeding in the Patas monkey. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02811 05 EA

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Aortic metabolism of plasma lipoproteins.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. W. Mahley — Head, Comparative Atherosclerosis and Arterial Metabolism Section — EA NHLI

D. L. Fry — Chief, Laboratory of Experimental Atherosclerosis — EA NHLI

K. H. Weisgraber — Senior Staff Fellow — EA NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.75	.25

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this study to determine which plasma lipoproteins are transported across the aortic endothelium, their mode of transport, and their fate in the aortic wall. Methodology for the in vitro maintenance of the aorta for periods of several hours has been developed.

PHS-6040
(12-75)

Project Description

Objectives: To determine 1) which classes of plasma lipoproteins are involved in aortic transport; 2) whether these lipoproteins are transported as intact macromolecules or hydrolyzed at the surface and components transported separately; 3) the fate of the lipoprotein components metabolized by the aorta.

Methods Employed: The in vitro transport method (described by Dr. D. L. Fry) is used to study aortic endothelial transport of the plasma lipoproteins under controlled conditions. Presently the dog is being used as the experimental model but we will soon extend this to the miniature swine. The components of canine plasma lipoproteins (VLDL, LDL, HDL_1 and HDL_2) are labeled with various radioisotopes. Initially we attempted to use ^{125}I as our protein tag. After exhaustive studies we conclude that it is impossible to limit the ^{125}I to the protein moieties, and variable amounts of lipids are labeled. The ^{125}I lipid label is unstable. We are now able to label the protein moieties of the canine lipoproteins with ^{35}S-methionine in vivo. At the same time we are able to label the phospholipids with ^{32}P-orthophosphate. ^{14}C-cholesteryl-esters and ^{3}H-free cholesterol moieties of these lipoproteins are labeled in vitro by the exchange method of Avigan. Uptake of label and the metabolism of the lipoproteins are followed by analysis of changes in the incubation media, examination of the aorta by direct isotope counting following oxygen combustion and by light and electron microscopic autoradiography.

Major Findings: Preliminary findings indicate the feasibility of this approach to the study of aortic endothelial transport and metabolism of plasma lipoprotein. Methodologic problems and validation of techniques continues to be a major component of this project. In addition to validation of the in vitro technique (described in a separate project report by Dr. D. L. Fry), methodology for the quantitation of four separate radioisotopes (^{14}C, ^{3}H, ^{35}S, and ^{32}P) has been established. The labeled plasma lipoproteins or a dried portion of the aorta following an in vitro transport study are placed in an oxygen combustion flask and ignited. ^{35}S-methionine which is the protein tag and ^{32}P which is the phospholipid tag are converted to inorganic sulfate and phosphate, respectively. These isotopes remain in the flask and are quantitated together by standard double label liquid scintillation counting. The flask is heated to drive off the tritium in the form of $^{3}H_2O$ and ^{14}C in the form of $^{14}CO_2$. The $^{14}CO_2$ is collected by bubbling the gas through a base converting it to an insoluble carbonate and the $^{3}H_2O$ is collected on a condenser in an ice bath. Isotope recovery is greater than 90% and modifications are being made to increase the efficiency of the method.

Significance to Biomedical Research and the Program of the Institute: It is agreed by most that 1) cholesterol within atheromata is derived largely from plasma lipoproteins and that 2) plasma lipoproteins can be detected within the same lesions. However, the mode of transport and the quantitative significance of lipoproteins in the lesions are far from clear. Serious

questions remain as to whether the lipoproteins cross the endothelial surface intact or whether they are hydrolyzed at the surface with only some of the components entering the tissue. Our in vitro approach to this problem should shed light on this most difficult problem of atherosclerosis research as well as add to our knowledge of lipoprotein metabolism.

Proposed Course of the Project: The project will be continued along the lines indicated above. It will also be extended to the miniature swine and nonhuman primates for comparative studies.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02812 07 EA

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Animal models for study of atherosclerosis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Mahley	Head, Comp. Athero. and Arterial Metabolism Sec.	EA NHLI
	D. L. Fry	Chief, Lab. of Experimental Atherosclerosis	EA NHLI
OTHER:	V. J. Ferrans	Visiting Scientist	OD-P NHLI
	J. E. Pierce	Chief, Sec. on Lab. Animal Medicine and Surgery	IR OD NHLI
	J. M. Phillips	Senior Veterinary Officer	IR OD NHLI
	D. K. Johnson	Chief, Veterinary Medicine and Surgery Sec.	VR DRS
	R. M. Jaffe	Staff Physician	CP CC
	R. L. Killens	Chief, Comparative Medicine Unit	VR DRS

COOPERATING UNITS (if any)

Veterinary Resources Branch, Div. of Research Services; Clinical Pathology, Clinical Center; and Colorado State University (Res. Contract #N01-HI-4-2903); Comparative Medicine Unit, Division of Research Services.

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Comparative Atherosclerosis and Metabolism Section

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this study to establish the proper dietary and metabolic conditions necessary to induce experimental atherosclerosis in various species which has characteristics similar to that of man.

In three species, the dog, miniature swine, and Patas monkey, this has been accomplished. The source of dietary fat has been found to have a profound effect in the type, distribution and severity of the atherosclerosis. The atherosclerosis produced by diets containing beef tallow, in addition to being severe, is associated with gross arterial thrombosis and occlusive vascular disease.

PHS-6040
(12-75)

Project Description

Objective: To determine the suitability of a variety of animals as models for atherosclerosis as compared to the human disease.

Methods Employed: The animal models which have been studied in varying detail are the Patas monkey, miniature pig, dog, rabbit, and rat. The experimental conditions under which the pathologic processes in these animals can be made to resemble those in man have been detailed in previous project reports. Briefly, the disease can be induced in the rabbit, pig, and monkey, by feeding diets high in cholesterol and fat (lard); whereas disease can be induced in the dog and rat to a comparable extent only if hypothyroidism is also induced. In dogs the types of dietary fats are varied and included cottonseed oil, pork lard, beef tallow, safflower oil and peanut oil.

Blood chemistries, which includes detailed lipoprotein studies, are monitored during the experimental period. At termination each animal is examined in detail using the standardized necropsy procedures as described previously. Topographic distribution and histologic characteristics of aortic, coronary, and peripheral arteries are compared to human atherosclerosis. The comparative human material is derived from young adults dying tramatic deaths, unselected hospital cases, and patients with documented types of hyperlipoproteinemia.

Major Findings: Dietary induced atherosclerosis in our principal animal models - the dog, swine and monkey - have features which are strikingly similar to that in man. The topographic distribution of lesions in all species formed a relatively characteristic and predictable pattern similar to that in man. Microscopic analysis showed early disease to be characterized by fatty streaking with only moderate intimal fibromuscular hyperplasia; whereas with longer duration of elevated serum lipids marked intimal fibromuscular hyperplasia occurred with the development of cell death, deposition of extracellular lipids and cholesterol crystals. Complicated disease appeared with the formation of atheromatous gruel under fibrotic caps, calcification and monocytic infiltration. The degree of disease in all the animals is directly correlated with the plasma cholesterol level and the appearance of a distinctive hyperlipoproteinemia. (Subject of progress report entitled, "Hyperlipoproteinemia and atherosclerosis. Changes in plasma lipoproteins and apolipoproteins induced by cholesterol feeding.")

The source of the dietary fat appears to be an important determinant of the distribution and morphologic characteristics of the disease in the dog. When beef tallow is substituted for pork lard as the principal dietary fat, the disease becomes much more severe and complicated in a shorter period of time with the development on many of the secondary complications as described in man. These complications include ulceration, thrombosis and embolism. Coronary artery disease with arterial occlusion and thrombosis is also accentuated when beef tallow is the source of dietary fat. The role of dietary fat, as a determinant of the type and severity of atherosclerosis, is being explored further in dogs and extended to swine, monkeys and rats.

The occurrence of gross arterial thrombosis in dogs on a tallow-cholesterol diet, but not on a cottonseed oil-cholesterol diet, prompted an extensive investigation of the effects of dietary fat on altered platelet function. In a colony of dogs maintained under contract with Colorado State University, various parameters of hemostatic function are being measured. These include *in vitro* response of platelets to aggregation, the *in vivo* measure of platelet survival and fibrinogen turnover. Preliminary data suggests that the source of dietary fat has a direct effect on certain of these hemostatic processes.

Significance to Biomedical Research and the Program of the Institute: The "atherosclerotic process" is, in fact, an ensemble of processes occurring at the cellular, physicochemical, biochemical, and biophysical level in the arterial intima. The purpose of this program is to identify as many of these fundamental processes as possible, establish which are relevant to those in man, and study these in great detail in the animal model, wherein the pertinent variables can be measured or controlled with a rigor not possible in man.

A clear definition of the role of diet in the development of atherosclerosis in man is of utmost importance.

Proposed Course of the Project: The pursuit of the above objectives will continue with our NIH collaboration.

Publications

1. Mahley, R. W., Nelson, A. W., Ferrans, V. J., and Fry, D. L. Thrombosis in association with atherosclerosis induced by dietary perturbations in dogs. *Science*, in press.

2. Mahley, R. W. Lipoproteins and arterial smooth muscle cells: Regulation of cellular metabolism by swine lipoproteins. International Workshop Conference on Atherosclerosis, London, Canada, Plenum Press, 1975, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02813 04 EA

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Tissue culture studies of aortic smooth muscle cells and skin fibroblasts: cell growth and metabolism in response to incubation with various lipoprotein classes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. P. Bersot	Clinical Associate	EA NHLI
OTHER:	R. W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLI
	D. L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.00	1.00	

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are designed to investigate the lipid metabolism and transport of molecular species through vascular smooth muscle and endothelial cells. In addition, the metabolism of normal and abnormal swine and human lipoproteins has been studied using skin fibroblasts obtained from normal humans and patients with dyslipoproteinemia. The primary objective is to understand the mechanism whereby plasma lipoproteins deliver lipids to cultured cells.

PHS-6040
(12-75)

Project Description

Objectives: 1) To study the effect of various lipoprotein classes upon aortic smooth muscle cell proliferation. 2) To study the effects of various lipoprotein classes upon smooth muscle cell and skin fibroblasts cholesterol metabolism. 3) To establish strains of swine and canine endothelial cells in culture.

Methods Employed: 1) Standard techniques were used in determining cell proliferation in response to various classes of lipoproteins. 2) Electron microscopy was used to establish that cells were similar to smooth muscle and endothelial cells as reported in the literature. 3) Cellular cholesterol production was assessed by measuring the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, the rate limiting enzyme in cholesterol synthesis. 4) Binding, uptake and degradation of iodinated lipoproteins were measured by standard techniques.

Major Findings: 1) Cultures of endothelial cells from swine and dogs have been maintained up to 7 generations in culture without alteration based upon electron microscopy of early and late generations. These cells lack numerous Weibel-Palade bodies in contrast to human umbilical vein endothelial cells in culture. 2) Swine smooth muscle cells bind, take-up and degrade ^{125}I-LDL but to a lesser degree than do skin fibroblasts. 3) In skin fibroblasts from normal human controls and swine aortic smooth muscle cells HMG CoA reductase activity was equally suppressed by human LDL and swine VLDL, LDL and HDL when each lipoprotein class was added in equal amounts with respect to cholesterol concentration. HDL suppressed enzyme activity only when added in 50-fold greater concentrations with respect to cholesterol.

Significance to Biomedical Research and the Program of the Institute: The differing responses of smooth muscle cells and fibroblasts in binding and degrading lipoproteins suggests that the ability (or lack of ability) of cells to metabolize exogenous lipid may play a role in the atherosclerotic process. Endothelial cell cultures will provide an opportunity to study the transport of plasma proteins across endothelial cells and the metabolism of lipoproteins by these cells.

Proposed Course of the Project: Further studies with smooth muscle cells will be done to elucidate the mechanism of lipoprotein cholesterol metabolism.

Publications

1. B. Greg Brown, Robert W. Mahley, and G. Assman: Swine Aortic Smooth Muscle in Tissue Culture: Cell Growth and Regulation of Cholesterol Synthesis in the Presence of Purified Swine Lipoproteins. Circulation Research, in press.

2. T. P. Bersot, R. W. Mahley, M. S. Brown, and J. L. Goldstein: Interaction of Swine Lipoproteins with the Low Density Lipoprotein Receptor in Human Fibroblasts. J. Biol. Chem., Vol.251, No. 8:2395-2398, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02814 01 EA

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Topographic analyses of endothelial surface permeability and atherosclerosis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLI
OTHER:	R. W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLI
	D. K. Johnson	Chief, Veterinary Medicine and Surgery Section	VR DRS
	J. E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery	IR OD NHLI
	J. M. Phillips	Senior Veterinary Officer	IR OD NHLI
	R. L. Killens	Chief, Comparative Medicine Unit	VR DRS

COOPERATING UNITS (if any)

Comparative Medicine Unit and the Veterinary Resources Branch, Division of Research Services

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.75	.75	3.00

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to compare the topographic patterns of increased endothelial surface permeability to the patterns of intimal lipid deposition of experimental atherosclerosis.

457

PHS-6040
(12-75)

Project Description

Objectives: To compare the topographic patterns of increased endothelial surface permeability to the patterns of intimal lipid deposition of experimental atherosclerosis.

Methods Employed: A systematized necropsy procedure and a standardized format was developed for arranging and fastening the opened arterial tree to reflective panels for optical scanning and analysis. This standardized format makes possible quantitative analysis of the topographic distribution of intimal Evans Blue dye accumulation (a measure of the permeability of the wall to plasma substances) and of intimal lipid deposition along the arterial tree of experimental animals. This standardized format may be expressed in a normalized coordinate system for automatic optical scanning and computer pattern analysis or may be used directly for comparative visual analysis. This format permits careful, detailed comparisons of areas of increased arterial wall permeability with areas of intimal lipid deposition at identical sites from animal to animal.

Major Findings: Application of these techniques to a recently completed study in experimental atherosclerosis in swine demonstrated that the disease pattern occurs with an extraordinary congruency from animal to animal. It was also shown that the patterns of increased endothelial permeability in normal animals are very similar to the patterns of intimal lipid deposition that occur in animals on an atherogenic regimen. At certain local sites, that were characterized by very high permeability in normal animals, a characteristic atheromatous lesion occurred which under the above standardized necropsy procedures appeared as a tri-colored lesion. These lesions were characterized by relatively normal surrounding intimal surface (white) a highly permeable (blue) center bordered by an annular but otherwise typical atheromatous plaque (red). With time, these lesions were shown to form mature atheromatous plaques in the abdominal aorta however in the thoracic aorta and subclavian artery these appear to mature more slowly. Some lesions in these areas appear to remain stationary for a matter of years maintaining their tri-color characteristics.

Significance to Biomedical Research and the Program of the Institute: The extraordinary congruency of the disease patterns from animal to animal in a given species, as well as to the patterns of permeability in the normal animal of that species represents a major finding of fundamental importance not only in linking the role of increased permeability to the subsequent development of this disease but also in permitting one to develop new strategies in experimental design to gain deeper insight into the associated atherogenic processes. The predictability of this process at given locations permits one to design studies to follow the sequence of events leading from the "earliest lesion" on through the complicated lethal atheromatous plaque. This capability was heretofore unavailable for lack of a standardized quantitative approach to the analyses of the disease topography and permeability topography. Moreover, the occurrence of the tri-color lesion provides the opportunity to study an apparent spectrum of atherogenic processes in a

very discrete anatomical region.

Proposed Course of the Project: This methodology has now been incorporated into the routine necropsy procedures for all of our animal colonies and will be used to assess the influence of a variety of dietary and other factors on the evolution of this important disease process. This methodology will make possible an approach to a number of fundamental questions: Why do some regions of moderate endothelial permeability progress only to the fatty streak lesions whereas others go on to mature atherosclerotic lesions? Why do certain local regions of extraordinarily high permeability appear to be relatively immune to lipid deposition? What is the sequence of events leading from a fatty streak to a lethal atheromatous plaque? How are the patterns of streaking and plaquing influenced by hemodynamic alterations, hematologic manipulations, dietary triglycerides, and exercise?

Publications

Fry, D. L. Hemodynamic forces in atherogenesis. In: Cerebrovascular Diseases, Tenth Princeton Conference, P. Steinberg, M.D., editor, New York: Raven Press, in press.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

The continuing goal of research in this laboratory is to elucidate the mechanisms of salt and water homeostasis in the kidney and other tissues in order to establish a basis for understanding the normal function as well as the understanding and treatment of disease processes. In order to achieve the goal we utilize a wide range of techniques, many of which have originated within our laboratory.

Isolated segments of renal tubules

Current advances in kidney physiology rely heavily on the direct study of individual nephron segments. The intact organ is too complex to be understood without anaylsis of its individual parts. One widely used method which originated in this laboratory is to dissect individual tubules and perfuse them in vitro. As detailed in previous reports, direct study of nephron segments in this manner has revealed a surprising diversity of function and uncovered a number of unexpected processes. Important examples include: 1) an active chloride transport system discovered in the thick ascending limb of Henle's loop, 2) the action of diuretics, which were found to inhibit chloride transport in this segment, 3) the interaction of vasopressin, prostaglandin, and the cyclic AMP system in the cortical collecting tubule, 4) the active sodium and potassium transport system in the cortical collecting tubule, 5) the organic acid and glucose transport system in proximal tubules, and 6) the complex mechanism governing fluid absorption in the proximal tubule.

Currently, we are studying acidification and its relationship to sodium transport. The generally accepted theory has been that reabsorption of sodium by proximal tubules is linked to secretion of hydrogen ions,implying that the two should vary concurrently. The principal fate of secreted hydrogen ions in proximal tubules is reaction with bicarbonate in the tubule lumen, resulting in bicarbonate reabsorption. Thus, if the theory is correct, the reabsorption of sodium and of bicarbonate should be mutually dependent. The theory has been challenged recently on the basis of micropuncture studies and is at present the subject of controversy. The issue is an important one in kidney physiology since sodium reabsorption and urinary acidification are vital processes that occur mostly in the proximal tubule. Previously, we found that sodium reabsorption was inhibited by the absence of bicarbonate, consistent with the theory. The present studies were intended to test the converse, i.e., whether bicarbonate transport is inhibited by the absence of sodium

transport. The studies were made possible by the recent invention by G. Vurek of the Laboratory of Technical Development of an ultra-micromethod for bicarbonate (or more strictly speaking for total CO_2). Our results support the classical theory. In proximal convoluted tubules bicarbonate reabsorption was greatly inhibited when sodium was absent or when sodium transport was inhibited by ouabain or by removal of potassium. Removal of chloride had no effect. The proximal straight tubule, which we previously found to differ from the convoluted tubule in some respects, was virtually identical in this regard. Thus, the straight tubule reabsorbed bicarbonate (although at a slower rate) and bicarbonate transport was inhibited in this segment when sodium or sodium transport was eliminated. Also, consistent with the theory, acetazolamide inhibited reabsorption of both sodium and bicarbonate.

On the other hand, in another part of the nephron, the cortical collecting tubule, there may be no link between sodium and hydrogen ion transport based on previous indirect studies. We will investigate this question directly in the near future, using the same methods as with the proximal tubules. Also, the studies of bicarbonate transport provide an introduction to the general study of urinary acidification. In order to continue in this line we require a practical method for measuring pH of tubule fluid. This is under development in collaboration with R. Steele of the Laboratory of Technical Development. In addition, we are setting up a method for elemental analysis of tubule fluid samples by electron probe. This provides the means to extend studies in isolated tubules to transport of magnesium, calcium and phosphate. Although the kidney obviously is important for the homeostasis of these substances, the processes involved are poorly understood.

Pathways of transport by proximal tubules

A major block to understanding salt and water transport in renal tubules as well as epithelia in general is ignorance of the exact transport pathways that are involved. It remains a matter for conjecture whether transported substances pass through the cells or between them, and for those that pass through cells whether they mix freely with the other cellular constituents. Dr. Spring, who recently joined the laboratory, proposes to study this question commencing with the study of chloride transport by Necturus proximal tubules. Preliminary to this study, he has developed microelectrodes which sense chloride ion activity. When these electrodes are inserted into a tubule cell they directly measure the ionic activity of intracellular chloride. Variations of intracellular chloride

activity as a function of extracellular chloride concentration will be used to determine the extent of transcellular chloride movement. In addition, the dependence of cell chloride activity on transmembrane potential difference will be assessed by systematically varying membrane potentials using the voltage clamp method. Also, cell membrane permeability to chloride may be determined directly, and the degree of coupling to sodium movement evaluated.

The experiments will utilize the isolated, perfused kidney of Necturus maculosus which is characterized by proximal tubules lying on the renal surface. The tubule cells, being large, are easy to impale with microelectrodes. Use of the perfused preparation enables precise control of the extracellular environment. A tubule perfusion method, previously devised for accurate measurements of transepithelial tracer sodium fluxes will be applicable to tracer chloride as well. By these means the transport pathway for chloride will be delineated, and eventually the pathways for other ions, as well.

The pathway for the transepithelial transport of fluid also remains speculative, awaiting the development of new experimental methods. There have been numerous efforts at developing mathematical models of fluid transport by epithelia. The data upon which many of the model calculations are based are derived from one of two types of experiments, as follows: 1) The tissue is exposed to an experimental condition, fixed and studied microscopically. 2) The tissue is subjected to a condition defined on the basis of the type 1) experiment to result in morphological changes and then functional changes are measured. "Leaky" epithelia which transport large volumes of salt and water have been extensively studied by these two approaches. Much of the transported salt and water was found to be shunted between the cells so that the tissue parameters were dominated by the shunt pathways. The experimental approaches outlined above failed to yield a definitive characterization of the shunt pathway. The microscopic approach failed because of fixation artefacts and delays which altered the tissue geometry. The second approach was inconclusive because forces which lead to morphological changes result in simultaneous changes in several tissue parameters obscuring cause and effect.

A new system is being developed for the present experiments designed to eliminate the morphological changes which accompany experimental manipulation. A combined optical-electrical system will control the volume of the intercellular shunt pathway during the imposition of physical forces. This so-called "interspace volume clamp" enables study of applied forces, uncomplicated by morphological changes. In brief, the intercellular spaces are filled with dye-containing solution

and the light absorption of a single space is measured quantitatively. The output of the photomultiplier tube is used as the input to a clamp circuit which passes sufficient current across the tissue to maintain the photomultiplier output at a constant value. When a physical force is varied across the tissue, transepithelial current flow is automatically modified to maintain interspace volume. Thus, the interplay of forces and flows will be determined in the absence of force-induced changes in geometry. Development of the optical system has been completed. A chamber was designed which allows electrical events and ion fluxes to be measured in a tissue viewed with high microscopic resolution. The tissue utilized initially for these experiments will be the gallbladder of Necturus maculosus. The large cells of the gallbladder are separated by readily discernable intercellular spaces. Various high molecular weight dyes for filling the interspaces have been tested and several appear promising.

Toad Bladder

Toad bladder epithelium has been used extensively as a model salt and water transporting system, resembling in this respect the distal nephron. It has been used in this laboratory to study the cellular mechanism of vasopressin and aldosterone control of salt and water excretion.

Earlier we found that vasopressin acts on responsive epithelial membranes to increase the production and accumulation within the cells of cyclic AMP, the intracellular "second messenger" of Sutherland and Rall. Cyclic AMP in turn elicits the permeability and transport response of the cell to the hormone. Subsequently, factors that affect the response to vasopressin have been examined. These factors include other hormones such as aldosterone, epinephrine, and prostaglandins, metabolic factors and chlorpropamide, a drug with considerable efficacy in the treatment of pituitary diabetes insipidus.

The epithelial cells of the toad bladder (and those of the mammalian distal nephron) are not morphologically homogenous. In the toad bladder, about 80% are "granular" and 15% are "mitochondria-rich." Functional correlates of the anatomical differences are not well established. Studies reported last year demonstrated that contrary to earlier reports, both cell types contain vasopressin-sensitive adenylate cyclase, the enzyme that catalyses the conversion of ATP to cyclic AMP. Current efforts are devoted to elucidating the intracellular control of cyclic AMP metabolism, and the mechanism of action of cyclic AMP on permeability and transport.

It has been proposed that cyclic AMP mediates the action of hormones by increasing the activity of protein kinases, i.e. enzymes that catalyse the transfer of the gamma phosphate of ATP to specific proteins. According to the theory, for which there is considerable evidence in some tissues, phosphorylation alters the function of the proteins. Cyclic AMP dependent protein kinase has been demonstrated in toad bladder epithelial cells and in mammalian renal cortex and medulla. The role of the protein kinase in these tissues is not established nor have specific protein substrates been identified. Last year we demonstrated that incubating isolated rabbit renal cortical tubules with parathyroid hormone or with cyclic AMP resulted in increased phosphorylation of four proteins. This was the first evidence that cyclic AMP stimulated protein phosphorylation in intact, transporting epithelial cells. (Previous studies utilized homogenates only.) The phosphorylated proteins were characterized by molecular size using polyacrylamide gel electrophoresis in SDS, but their role in the response to parathyroid hormone remained to be determined.

The studies were continued this year by examining phosphoprotein metabolism in toad bladder epithelial cells. Protein kinase from the 100,000 x g supernatant solution of homogenates was purified by chromatography on DEAE cellulose as well as by gel filtration and hydrophobic chromatography. Phosphoprotein phosphatase (an enzyme that catalyses the removal of phosphate from phosphoproteins) coeluted with the protein kinase from DEAE cellulose and was only partially separated from the protein kinase in other chromatography systems. The phosphoprotein phosphatase activity was not stimulated by cyclic AMP. The principal endogenous protein substrate for the protein kinase was a 50,000 molecular weight protein found in the cytosol. We plan next to isolate plasma membranes and study their phosphoprotein metabolism since membrane proteins are more likely than those from the cytosol to play a major role in transepithelial transport.

Cyclic 3',5' guanosine monophosphate (cyclic GMP) is involved in the response of some tissues to hormones, usually with an effect antagonistic to cyclic AMP. There is cyclic GMP in renal tissue and in urine. In order to investigate the role of cyclic GMP, we measured its concentration in toad bladder epithelial cells. It was two orders of magnitude lower than the concentration of cyclic AMP. The concentration of cyclic GMP increased by 50% 5 minutes after 10^{-4}M carbamyl choline was added to the epithelial cells but this effect did not occur when the bladders were pretreated with aldosterone. Vasopressin and epinephrine on the other hand, did not affect cyclic GMP concentration. Carbamyl choline also inhibited sodium transport(short-circuit current). The results with carbamyl choline, although of interest, are particularly

difficult to interpret since the drug causes the smooth muscle of toad bladders to contract markedly. Further studies will be required to distinguish any primary effect on the epithelial cells from effects secondary to the muscular contraction.

Cyclic nucleotide phosphodiesterase catalyses the degradation of cyclic AMP and cyclic GMP and therefore might conceivably moderate the response to hormones. In order to study this question, we looked for protein activators or inhibitors of the phosphodiesterase such as have been described in several other tissues. None were found. Cyclic nucleotide phosphodiesterase was present in at least two discrete forms which we separated by DEAE cellulose column chromatography. The first peak had a high K_m for cyclic AMP and a low K_m for cyclic GMP. The second peak had a low K_m for cyclic AMP. We previously found that aldosterone inhibited phosphodiesterase. We will next test whether both forms of enzyme are inhibited by aldosterone and whether there is a difference in the enzyme between the granular and mitochondria-rich cells.

Cholera toxin activates adenylate cyclase (which catalyses the production of cyclic AMP) in many tissues in addition to the intestine. We are studying how the toxin interacts with the enzyme in order to better understand control of the enzyme. Adenylate cyclase is located in the serosal plasma membrane in toad bladder, as in the gut. Cholera toxin affects the enzyme more readily when added to the solution bathing the mucosal surface, despite the fact that the enzyme is located in the membrane on the opposite side of the cell. We initially believed that this reflected intrinsic differences in the plasma membranes at the two surfaces of the epithelial cells. More recently, however, we found in autoradiographs utilizing I^{125} cholera toxin that the toxin did not reach the serosal surface of the cells when placed in the solution on that side of the bladder but was bound by some barrier in the supporting stroma. We suspect that the polarity of cholera toxin effect in the gut has a similar explanation. The ganglioside GM_1 is a glycolipid that binds avidly to cholera toxin. It has been generally believed that ganglioside GM_1 in plasma membranes acts as the receptor for the toxin. Our results using toad bladders are consistent with this conclusion. Thus, we found that toxin added along with ganglioside GM_1 was inactive. Presumably, the exogenous ganglioside bound the toxin, preventing its access to the ganglioside in the cell membrane. Further, the effect of cholera toxin added to the solution on the mucosal surface of the bladder was enhanced by adding ganglioside GM_1 to the solution on the serosal surface of the bladder, presumably because the additional ganglioside became incorporated in the cell membranes. An unexpected finding was that vasopressin, added to the solution on the serosal surface of the bladder, altered the response of the cells to cholera toxin

added to the mucosal solution. The results were as follows: a) vasopressin enhanced the binding of ^{125}I-cholera toxin to the cells and also enhanced the effect of the toxin. b) Vasopressin accelerated the response of the bladder to toxin so that it was evident after two hours compared to the usual delay of 6 hours. c) Cholera toxin contains 2 subunits, one which binds to the ganglioside receptor and one of which ("A") activates adenylate cyclase. Ordinarily, both subunits are necessary for activity. Vasopressin sensitized the epithelial cells, however, so that they responded to the "A" fragment of toxin alone.

Control of volume of nucleated red blood cells

Novel transport systems that regulate the volume of cells were first identified and characterized in studies of avian erythrocytes in this laboratory. Subsequently, similar systems have been identified in other tissues and their study has had important implications both for normal cell homeostasis and for the understanding of pathological derangements of cell volume. Although the processes involved have been extensively characterized, the basic mechanisms are not completely understood and remain a subject of continued study in our laboratory.

We previously found that when duck erythrocytes were placed in hypotonic media, they enlarged immediately (owing to osmosis), then shrank rather rapidly back towards their original volume. The latter constituted a volume regulatory phase (cell shrinkage). The cell shrinkage was found to be caused by increased permeability of the cells to potassium. Potassium leaked out of the cells, down its electrochemical gradient, followed by anions and water. When this process had caused the volume to return to normal, the permeability to potassium also returned to normal.

There was an analogous response when cells were placed in hypertonic media. They shrank immediately (owing to osmosis), then under appropriate conditions swelled gradually back towards their original volume. In this case (cell enlargement) both sodium and potassium fluxes into and out of the cells increased greatly. Certain catecholamines caused similar increases in cation fluxes followed by cell enlargement even in the absence of any preliminary osmotic stimulus. Cells enlarged because sodium and potassium movements, although accelerated in both directions were slightly greater into the cells. This resulted in net cellular uptake of cations, followed by anions and water. It was of special interest that cell enlargement did not occur unless the concentration of potassium in the medium, $[K]_o$, was 2.5 mM or above. Below this

concentration, although the increased cation fluxes occurred, the cells did not enlarge. The reason for the critical dependence on $[K]_o$ remains unknown, but is of obvious importance and is the subject of continuing investigation.

We had also found that the response to norepinephrine involved generation of cyclic AMP, but that the response to hypertonicity did not. In other respects the responses were similar, however, and the question remained whether an identical effector was involved, the effector being triggered in one instance by catecholamines (via cyclic AMP) and in the other instance by hypertonicity. The specific characteristics of the increased cation fluxes induced by norepinephrine had been studied in detail. In brief, the results were that when $[K]_o$ was 2.5 mM, norepinephrine stimulated all the cation fluxes (Na influx and efflux and K influx and efflux). When $[K]_o$ or $[Na]_o$ was zero, only Na efflux was stimulated. Further, the efflux of sodium and potassium were saturating functions of both $[K]_o$ and $[Na]_o$. This mutual dependence implied some kind of co-transport mechanism, but it has proved difficult to conceive of a simple co-transport model which encompasses all of the findings. This point aside, it was logical that, if the same effector is activated by norepinephrine and hypertonicity the sodium and potassium fluxes that result should be identical. We tested this point in the present year by measuring the sodium and potassium fluxes activated by hypertonicity and their dependence on external sodium and potassium. The results were identical to those following norepinephrine, adding support to the theory that the same effector is involved in both cases.

Although differing in other respects cell shrinkage and cell enlargement have in common that K efflux is activated in both cases. In order to test whether the same mechanism is involved, the effects of different inhibitory conditions were tested on both responses. Furosemide, low $[K]_o$, and replacement of Cl by SO_4 either inhibited the K efflux associated with cell enlargement without any effect on K efflux associated with cell shrinkage or affected the former process to a greater degree. Therefore, cell enlargement and cell shrinkage most likely involve different mechanisms.

Measurements carried out on single red blood cells

Last year we developed a novel method for studying single red blood cells of Amphiuma by immobilizing the cells in the tip of a micropipet. By this method it was possible to measure transmembrane potential, resistance, and isotope fluxes of the red cell more directly than in the past. We found the permeability to ^{36}Cl to be high. This was attributed both to Cl self-exchange and net permeation, based on measurements of

^{36}Cl flux during voltage clamping. The self-exchange was believed to be carrier mediated. In mammalian erythrocytes, the disulfonic stilbene derivation, "SITS" inhibits an anion mechanism responsible for self-exchange. In the present studies we found that "SITS" greatly inhibited ^{36}Cl flux across Amphiuma red blood cells consistent with the theory. We have also extended measurements of the membrane potential and resistance reported in a preliminary form last year, and we are now completing extensive control studies which validate this new experimental method. While the method until now has been used only for red blood cells, we anticipate that it will prove valuable in the future for the study of other types of cells, as well.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01201-02 KE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of cholera toxin on toad urinary bladder

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Joseph S. Handler, M. D. Head, Section on Membrane Metabolism KE NHLI

OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney & Electrolyte Metabolism

SECTION

Membrane Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	0.8	2.0

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this study to examine the activation by cholera toxin of toad bladder adenylate cyclase, the enzyme activated by the hormone vasopressin. The effects of ganglioside, GM_1 (the receptor for cholera toxin), of vasopressin, and of the binding subunit of the toxin will be studied.

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PHS-6040
(12-75)

Project Description

Objectives: It is generally accepted that the toxin of Vibrio cholera (choleragen) acts on intestinal epithelial cells and other tissues by stimulating adenylate cyclase activity. The resulting elevation of intracellular cyclic AMP levels is responsible for the typical response to the toxin, secretion of electrolyte and water in the small intestines, and in other tissues, responses resembling those elicited by specific hormones which stimulate adenylate cyclase. Recent reports indicate that choleragen binds to a specific glycolipid, the ganglioside GM_1 which is the binding site or receptor for choleragen in cell membranes. Once choleragen is bound to cell membranes, a temperature and time dependent reaction appears to be required for adenylate cyclase activation. It is the purpose of this study to examine the effect of choleragen on the toad urinary bladder. Normally, in vivo, vasopressin activates adenylate cyclase in the basal-lateral (blood surface) plasma membrane of the epithelial cells of the toad bladder. The resultant elevation of the intracellular concentration of cyclic AMP causes an increased rate of active sodium transport by the bladder and an increase in the permeability of the bladder to water. The objectives of the study are to gain information about the role of the epithelial cell plasma membrane in the activation of adenylate cyclase, and about the mechanism of action of the toxin.

Methods: All experiments were performed with paired hemibladders incubated in amphibian Ringer solution with agents of interest for periods of 30 min. to 24 hrs. Experiments were ended by measurement of adenylate cyclase activity in homogenates of the mucosal epithelial cells, by measuring the amount of radioactively labelled cholera toxin bound to the epithelial cells, or by measuring the water permeability or sodium transport response of the bladder to vasopressin, to cyclic AMP, or to theophylline. Choleragen was labelled with ^{125}I using the iodine monochloride method. The binding subunit (B fragment) and the active subunit (A fragment) of choleragen were prepared in 6 M urea, pH 4. glycine buffer using gel filtration chromatography.

Major Findings

1) Incubation with choleragen 0.5 µg/ml on the mucosal surface or 5 µg/ml on the serosal surface for 18 hrs. results in activation of epithelial cell adenylate cyclase activity assayed in homogenates. The activation is more marked in assays performed in the presence of low concentrations of GTP. The adenylate cyclase response to vasopressin is also enhanced and the apparent activation constant for vasopressin is reduced. In contrast, the stimulation of adenylate cyclase by fluoride is reduced by choleragen. These observations confirm the interpretation of earlier experiments in which incubation with choleragen

increased water permeability and sodium transport response of the bladder to vasopressin and to theophylline (see Z01 HL 0120101 KE, last year's report).

2) The ganglioside GM_1 is known to bind avidly to the binding subunit of choleragen. It is believed to be present in most cell membranes and to serve as the receptor for choleragen. Incubation of choleragen with ganglioside GM_1 for 30 min. before addition of the mixture to the solution bathing the mucosal or serosal surface of bladder results in reduced response to the toxin, supporting the concept that the ganglioside binding site in choleragen is involved in choleragen action on the bladder from both mucosal and serosal surfaces. We have confirmed the observation that (see Z01 HL 01201-01 KE) incubation of the serosal surface of the bladder with ganglioside GM_1 results in enhanced responsiveness of the bladder to choleragen added to the mucosal surface. We have not been able to demonstrate that incubation of the mucosal surface with ganglioside GM_1 results in enhanced responsiveness to toxin added to the mucosal or to the serosal surface. A possible interpretation of these results is that ganglioside GM_1 can enter the cell only from the serosal surface and that it is incorporated into the plasma membrane on the mucosal surface of the cell, perhaps by diffusion within the plasma membrane from the serosal surface. The fact that choleraagen added to the solution bathing the mucosal surface activates adenylate cyclase, an enzyme in the cell membrane on the serosal surface of the cell may also indicate movement of material from one surface of the cell to the other within the plasma membrane.

3) Incubation of the bladder with the B fragment (the binding segment) of choleragen reduces the response of the bladder to whole choleragen. The A fragment of choleragen, which is thought to be the active subunit because it activates adenylate cyclase in broken cell preparations, is without effect on the intact bladder. Presumably it is without effect because it cannot bind to and enter the epithelial cells since it lacks the B fragment. These results resemble those found in other tissues.

4). There is a considerable delay between binding of choleragen to the bladder and the activation of adenylate cyclase. Bladders exposed to mucosal choleragen for 30 minutes and assayed for choleragen effects 18 hrs later have a response to choleragen similar to that of bladders exposed to choleragen for 18 hours, indicating that maximal effective interaction between choleragen and bladder is achieved in about 30 min. The effect of choleragen is not evident before six hours of incubation. The nature of the rate delaying step in choleragen action is not known. The delay can be shortened to two hours by the concurrent addition of high concentrations of vasopressin to the serosal surface. Vasopressin has no effect on the response to choleragen added to the

serosal surface. The concurrent addition of vasopressin to the serosal solution and A fragment to the mucosal solution results in a choleragen-like effect of A fragment, which as mentioned, has no effect on the bladder in the absence of vasopressin. It is generally accepted that vasopressin elicited changes in the permeability of the bladder are the result of changes in some characteristics of the plasma membrane on the mucosal surface of the epithelial cells. It is possible that the vasopressin elicited changes in the mucosal plasma membrane enable the A fragment to permeate the plasma membrane and also accelerates the response of the bladder to choleragen. Preliminary experiments with ^{125}I-choleragen indicate that vasopressin increases the binding of choleragen added to the mucosal solution.

Significance to biomedical research

These studies have revealed several new properties of the toad urinary bladder, a membrane of epithelial cells that responds to hormones and has transport properties similar to those of the distal nephron of man. In addition, it appears that many characteristics of the action of cholera toxin on the toad urinary bladder are common to the action of the toxin on the small intestines and may yield additional experimental approaches to the study of the naturally occurring infectious disease.

Proposed course

In order to study the markedly greater sensitivity of the bladder to mucosal choleragen than to serosal choleragen (see last year's report Z01 HL 02101-01 KE), autoradiography of the intact bladder, and in other experiments the binding to epithelial cells of ^{125}I-choleragen added to the mucosal solution will be compared to that of ^{125}I-choleragen added to the serosal solution.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01202-02 KE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Control of protein phosphorylation in toad urinary bladder

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gordon J. Strewler, M.D.	Clinical Associate	KE	NHLI
OTHER:	Joseph S. Handler, M.D.	Head, Section on Membrane Metabolism	KE	NHLI
	Jack Orloff, M.D.	Director, IR		NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to define the role of protein phosphorylation in the action of vasopressin on the toad bladder. Current interests are: 1) the intracellular location and functional characteristics of cyclic AMP dependent protein kinases 2) The role of phosphoprotein phosphatases in the control of phosphoprotein metabolism. 3) The functional significance of the principal phosphoproteins, particularly their role in transport.

PHS-6040
(12-75)

Project Description:

Objectives:

Previous studies in this laboratory have established that the actions of the hormone vasopressin on sodium transport and on water permeability in toad urinary bladder and in the renal collecting tubule of the rabbit are mediated by increases in the cellular levels of cyclic AMP. In certain other tissues the effects of cyclic AMP are thought to be the result of protein phosphorylation catalysed by cyclic AMP dependent protein kinases. The state of phosphorylation of proteins is determined by the activity of protein kinases and phosphoprotein phosphatases. We have previously shown effects of cyclic AMP on protein phosphorylation in homogenates of toad bladder epithelial cells. The subcellular location of protein kinase activity and of protein substrates for phosphorylation has been partially defined. Among phosphoproteins affected by cyclic AMP the principal is a 50,000 dalton soluble protein. Cyclic AMP stimulates its phosphorylation and in such preparations may also enhance slightly its dephosphorylation.

The present objectives of this study are: 1) to purify partially the enzymes responsible for phosphoprotein metabolism: a protein kinase and a phosphoprotein phosphatase; 2) using these purified enzymes, to determine whether protein substrates for phosphorylation and dephosphorylation exist in subcellular fractions, especially the plasma membrane that is believed to be the rate limiting permeability barrier that is altered by vasopressin; 3) to elucidate the functions of the major phosphoproteins, searching in particular for a role in hormonal control of transport in this tissue.

Methods:

Protein kinase activity is assayed using the filter disk method of Corbin and Reimann (Methods in Enzymology 38:287, 1974). Phosprotein phosphatase activity is measured by the method of Maeno and Greengard (J. Biol. Chem 247:3269, 1972). Phosphoproteins labelled with ^{32}P from $\gamma^{32}P$ ATP in the presence of an endogenous protein kinase are separated using gel electrophoresis in SDS. Subcellular fractions are prepared employing differential centrifugation and sucrose density gradient centrifugation. Purity of fractions is monitored with marker enzyme assays as described prevously.

Major findings:

A single peak of cyclic AMP dependent protein kinase activity elutes from DEAE cellulose. It has been purified further using

gel filtration and hydrophobic chromatography. This enzyme is stimulated about five fold by cyclic AMP and has some characteristics of a type II kinase (Corbin, J.D. et al, J. Biol. Chem. 250:218, 1975). Phosphoprotein phosphatase activity coelutes with the protein kinase from DEAE cellulose. It is only partially separated from the protein kinase in other chromatography systems. Using hydrophobic chromatography three different peaks of phosphatase activity are demonstrated. Phosphoprotein phosphatase activity is inhibited by phosphate and fluoride, and is not stimulated by 10^{-6} M cyclic AMP. The partially purified cyclic AMP dependent protein kinase is capable of incorporating phosphate into endogenous proteins. The principal among these is the 50,000 dalton soluble protein, which elutes from DEAE cellulose with protein kinase. This 50,000 dalton protein does not migrate on SDS polyacrylamide gels with purified rat brain tubulin, and given the remarkable genetic conservatism for tubulins, this suggests it may be different also from toad bladder tubulin, for which a role in control of water permeability has been suggested.

It has not been possible to demonstrate protein kinase activity or a phosphorylated protein in a plasma membrane fraction from the toad urinary bladder. However, high levels of ATPase activity in this fraction, preclude a definitive statement regarding cyclic AMP dependent plasma membrane phosphorylation.

Significance to biomedical research:

At present the significance of these preliminary findings is uncertain. While there is clearly active metabolism of phosphoproteins under the control of cyclic AMP in the toad urinary bladder epithelium its contribution to the physiological effects of vasopressin on transport remains to be determined.

Proposed course:

1. Further attempts will be made to prepare a well-characterized plasma membrane fraction from toad bladder epithelial cells that allows further study of phosphorylation of protein in plasma membranes. Control of phosphorylation in this fraction by cyclic AMP will be studied.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01214-01 KE

PERIOD COVERED
July 1, 1975 to June 30, 1976.

TITLE OF PROJECT (80 characters or less)
Cyclic nucleotide metabolism in toad urinary bladder

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. K. Sahib, Ph.D.	Visiting Scientist KE	NHLI
OTHER:	J. Orloff, M. D.	Director, Intramural Research	NHLI
	J. S. Handler, M. D.	Head, Section on Membrane Metabolism KE	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with measurement of cyclic 3',5' guanosine monophosphate in the epithelial cells of the toad urinary bladder and an examination of changes in the cyclic 3',5' guanosine monophosphate content elicited by agents that affect transport and permeability properties of the bladder. A second phase of the study is devoted to characterising cyclic nucleotide phosphodiesterase activity in the bladder and the effect of aldosterone on the enzyme.

478

PHS-6040
(12-75)

Project description:

a. Studies on cyclic 3',5'-guanosine monophosphate (cGMP)

Objective

3':5'-cyclic guanosine monophosphate is found in normal urine and mammalian kidney but the role (if any) of the nucleotide in regulation of kidney function remains to be determined. No information is available about the basal level or the role of the nucleotide in toad urinary bladder, a transporting, hormone-responsive membrane of epithelial cells, analogous in many respects to the mammalian distal nephron. It is the purpose of this study to adapt the available radio-immunoassay for cGMP to measurement of cGMP in toad bladder epithelial cells and to study the effect of regulatory agents, primarily hormones, on the content of cGMP in the tissue in order to elucidate the role of the nucleotide.

Methods

Epithelial cells were collected from urinary bladders by scraping, and were placed in liquid nitrogen. The frozen cells were extracted with 5% trichloracetic acid. The pellets were analyzed for protein and the supernatant solution was processed for radio-immunoassay by extraction with 5 vols of ether, lypophilisation of the aqueous phase and succinylation of the lyphilised residue.

In order to evaluate the effect of different physiologic and pharmacologic agents on the cGMP levels of the cells, the cells were incubated with different agents in Ringer solution and then processed for radio-immunoassay of cGMP as described above. The effect of aldosterone was examined by incubating the hemibladders with or without aldosterone for 16 hrs and then collecting the epithelial cells.

The effect of carbamyl choline and related agents on sodium transport was monitored by following the effect of these agents on potential difference and short-circuit current of the tissue.

Major Findings:

cGMP level in toad bladder epithelial cells was found to be two orders of magnitude lower than that of cAMP. Incubation with 10^{-4} carbamyl choline for 5 min. increased cGMP levels by 50-100% over the basal level. Norephinephrine and vasopressin did not elicit changes in cGMP content. Incubation of bladder with 10^{-7} M aldosterone inhibits the response to carbamyl choline.

Carbamyl choline inhibits Na^+ transport as is evident by its lowering of potential difference and short-circuit current by 50% for 10-15 min. Carbamyl choline also causes contraction of the smooth muscle of the bladder. It is not possible to judge whether the effect of carbamyl choline on short-circuit current is a direct effect of the drug on the epithelial cells or is secondary to the muscle contraction.

Proposed course of project

The effect of carbamyl choline on short-circuit current will be examined in control and aldosterone treated bladders in order to determine whether aldosterone alters the effect of carbamyl choline on sodium transport as it does the effect on cGMP levels. The cGMP aspect of the project will not be pursued further unless a physiologic role for cGMP becomes apparent.

b. Studies on cyclic nucleotide phosphodiesterases

Previous studies in this laboratory have led to the interpretation that the permissive effect of aldosterone on biochemical and physiological responses of the toad bladder to vasopressin is a consequence of a steroid induced decreased rate of degradation of cAMP by cAMP phosphodiesterase. In those studies, high and low K_m cyclic AMP phosphodiesterase activity was found to be about 20% lower in aldosterone treated tissue although cAMP levels were at least an order of magnitude higher. It is the purpose of this investigation to characterise cyclic nucleotide phosphodiesterases of toad bladder epithelial cells further, and to look for evidence of an activator of phosphodiesterase.

Methods:

Extracts of toad urinary bladder epithelial cells were prepared by homogenizing epithelial cells scraped from bladders of perfused toads in 25mM tris HCl buffer, pH 8.0, containing 0.5mM $MgCl_2$ and 0.1mM dithiothreitol in a polytron. The homogenates were centrifuged at 100,000 xg for 1 hr in the cold. The supernatant was used to study the enzyme.

Major findings

Prolonged dialysis of the tissue extracts against 0.5mM EGTA inhibited both high as well as low Km phosphodiesterase by about 50%. Addition of Ca^{++} plus undialysed cell extract, however, failed to stimulate the enzyme activity. On the one hand inhibition of phosphodiesterase activity on dialysis against EGTA

suggests the presence of Ca^{++} dependent activator as described in some other tissues, while on the other hand failure to demonstrate activation by Ca^{++} or Ca^{++} plus undialysed extract leaves the question open regarding presence of a calcium dependent activator.

Phosphodiesterases could be resolved into at least two discrete forms on DEAE cellulose column chromatropgraph depending upon their elution by different salt concentrations. Peak I was found to have high K_m for cAMP and very low K_m for cGMP while peak II showed high affinity for cAMP. Both the peaks revealed normal Michaelis-Menten kinetic behavior suggesting minimal cross contamination.

About 50% inhibition of cAMP phosphodiesterase was observed in aldosterone treated cells when the enzyme was assayed in intact cells rendered permeable to exogenous cAMP by toluene treatment. Apparently, the aldosterone induced changes in the enzyme are more evident in this assay system in which the organization of cell proteins is disrupted minimally compared to homogenization.

Proposed course of the project

The DEAE cellulose elution profile of cyclic nucleotide phosphodiesterase from cells incubated with aldosterone will be compared to that of the control cells. Epithelial cells isolated from aldosterone treated and control bladders will be partitioned into mitochondria-rich and granular cells using density gradient centrifugation, and cyclic nucleotide phosphodiesterase assayed in each type. Each type of cell will be analysed for an activator or inhibitor of cyclic nucleotide phosphodiesterase.

Significance to biomedical research

Endocrine regulation of transport processes in epithelial membranes may involve cGMP and certainly is affected by cAMP phosphodiesterase. The results of this study should clarify understanding of the regulatory process.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01206-02 KE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Urinary acidification and fluid absorption by proximal straight tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thurman D. McKinney, M.D.	Research Associate KE NHLI
	Maurice B. Burg, M.D.	Chief, Laboratory of Kidney & Electrolyte Metabolism KE NHLI
OTHER:	Roderic Steele Ph.D.	Laboratory of Technical Development TD NHLI

COOPERATING UNITS (if any)

Laboratory of Technical Development

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Renal Mechanism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is to define the mechanisms of bicarbonate and fluid absorption in individual isolated perfused nephron segments from rabbit kidneys. The present areas of interest concern measurement of bicarbonate and fluid reabsorption in proximal straight tubules and their dependence on sodium, chloride, bicarbonate and carbonic anhydrase.

PHS-6040
(12-75)

Project Description

The proximal straight tubule contributes significantly to bicarbonate and fluid absorption by the kidney. Bicarbonate reabsorption is felt to occur primarily via hydrogen secretion and contributes to fluid absorption in this nephron segment. In order to further elucidate the mechanisms involved in these processes we have:

1. Evaluated the effect of altering bicarbonate reabsorption on fluid reabsorption.

2. Further defined some of the characteristics of bicarbonate reabsorption.

Methods

1. The method of isolation and perfusion of tubule segments from rabbit kidneys developed in this laboratory has been described in previous reports.

2. Total CO_2 content in nanoliter samples was measured by a microcalorimétric method (picapnotherm), also, as previously described.

Major Findings

When bicarbonate was omitted both from the perfusate and bath, fluid absorption decreased 20%. When it was omitted only from the perfusate, but remained in the bath, fluid absorption did not change significantly. With bicarbonate both in the perfusate and bath the carbonic anhydrase inhibitor acetazolamide caused fluid absorption to decrease by 23% with 10^{-5}M of the drug and 36% with 10^{-3}M.

Even when the tubules were perfused and bathed in solutions free of bicarbonate, acetazolamide still had an effect. Under these conditions 10^{-4}M of the drug caused fluid absorption to decrease by 32%.

Thus, a significant part of fluid absorption in the proximal straight tubule is dependent on bicarbonate. However, even in the absence of bicarbonate significant fluid absorption continues and is inhibited by acetazolamide, indicating an effect of this carbonic anhydrase inhibitor on fluid (sodium) reabsorption independent of ambient bicarbonate.

With 25mM bicarbonate in both the perfusate and bath, the total CO_2 content in collected tubule fluid decreased to 8.2mM, at slow rates of perfusion confirming that there is reabsorption of bicarbonate and acidification in proximal straight tubules.

When the tubules were perfused at slow rates with bicarbonate present in the bath, but not in the perfusate total CO_2 appeared in the collected fluid. At slow flow rates the mean concentration was 7.7mM. Since the concentration of total CO_2 in the collected fluid was virtually the same at slow flow rates whether bicarbonate was present in the perfusate or not, a steady state concentration of total CO_2 evidently was reached.

Omission of potassium from the bath or addition of ouabain (10^{-5}M) to the bath caused the total CO_2 content in the collected fluid to rise to 19mM (as compared to 8mM in the control state) at the slow rate of perfusion, indicative of inhibition of transport of total CO_2.

Substitution of lithium or tetramethyl ammonium for sodium in the perfusate and bath also caused the concentration of total CO_2 in the collected fluid to rise markedly.

On the other hand, substitution of nitrate for chloride in the perfusate and bath caused no change in the concentration of total CO_2 content in the collected fluid.

These results indicate that total CO_2 (bicarbonate) reabsorption is dependent on sodium reabsorption, but is not dependent on chloride.

Proposed Course of Project

Additional studies will include:

1. The effect of sodium substitution by choline on the total CO_2 concentration.

2. Measurement of the absolute rate of total CO_2 reabsorption and the effect of acetazolamide and the substitution of other cations for sodium on this rate.

3. Development of a glass pH micro electrode in collaboration with Dr. Steele. With this apparatus it should be possible to elucidate the mechanism of acidification and bicarbonate reabsorption.

4. Similar studies will be performed in cortical collecting tubules, where there is reason to believe that bicarbone transport may not be sodium-dependent.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01208-02 KE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mechanism of salt and water transport by proximal renal tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Maurice B. Burg, M.D.	Chief, Laboratory of Kidney and Electrolyte Metabolism	KE NHLI
OTHER:	Nordica Green	Chemist	KE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Renal Mechanisms

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

SUMMARY OF WORK (200 words or less - underline keywords)

The general objective is to characterize the transport function of individual nephron segments in order better to understand kidney function. Renal tubules are dissected from rabbit kidneys and perfused in vitro. In the present studies the effect of acetazolamide, a diuretic and carbonic anhydrase inhibitor was tested on fluid and salt transport by proximal convoluted tubule.

PHS-6040
(12-75)

Objectives:

The proximal nephron reabsorbs approximately 50% of the glomerular filtrate. The mechanisms by which this occurs have been only partially characterized. Earlier work on this project (see report for July 1, 1974 to June 30, 1975) confirmed that sodium is actively reabsorbed providing the primary driving force for fluid transport. The process is complicated, however, and involves coupling between transport of sodium and various other solutes as well as coupling to cellular metabolism, as a source of energy. Among the solutes to which sodium transport is coupled are hydrogen ions and bicarbonate.

Acetazolamide is a drug which is a carbonic anhydrase inhibitor and diuretic known to act in the proximal convoluted tubule. Since it affects transport of bicarbonate and hydrogen ions, as well as sodium, an understanding of its mechanism of action might help elucidate the relationship between the transport of these various solutes.

Methods:

The rabbit isolated perfused proximal convoluted tubule preparation developed in this laboratory and described in previous reports was used to analyze the transport processes. The rate of absorption of fluid was measured using inulin-C^{14} as a volume marker. Since the concentration of sodium does not change during fluid absorption, the method provides a measure of sodium transport, as well.

Major Findings:

1. Acetazolamide partially inhibited fluid absorption in concentrations as low as 10^{-6}M. Inhibition was 35% at 10^{-5}M which was the maximal effect. Therefore, this concentration was used for most of the remaining studies.

2. The drug acted when placed in either the lumen or bath, but the effect was slightly greater from the lumen side.

3. As previously reported, elimination of bicarbonate from the perfusate and bath caused the rate of fluid absorption to decrease. There was no additional decrease when acetazolamide (10^{-5} or 10^{-4}M) was added, suggesting that the effect of the drug on sodium and fluid transport is mediated via its effect on bicarbonate transport.

Significance to biomedical research:

Excretion of bicarbonate and sodium are major regulatory functions of the kidney, which remain incompletely understood despite considerable previous experimental effort. The present studies afford direct evaluation of the important variables.

Proposed course:

To test further the effect of acetazolamide on the cortical collecting tubule, a nephron segment which has a mode of sodium transport differing from the proximal tubule.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01215-01 KE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Bicarbonate transport by proximal convoluted tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Maurice B. Burg, M.D.	Chief, Laboratory of Kidney and Electrolyte Metabolism	KE NHLI
OTHER:	Noridca Green	Chemist	KE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Renal Mechanisms

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	3/4	3/4

SUMMARY OF WORK (200 words or less - underline keywords)

The general objective is to characterize the transport function of individual nephron segments in order better to understand kidney function. Renal tubules are dissected from rabbit kidneys and perfused in vitro. In the present studies bicarbonate transport by proximal convoluted tubules was measured, using a newly developed micro calorimeter to assay total CO_2 content. The principal conclusion is that bicarbonate reabsorption is dependent on simultaneous transport of sodium.

PHS-6040
(12-75)

Project description

Objective

It has been generally believed that reabsorption of sodium by proximal renal tubules is linked to secretion of hydrogen ions so that the two vary concurrently and affect each other. The principal fate of secreted hydrogen ions in proximal tubules is reaction with bicarbonate in the tubule lumen, resulting in bicarbonate reabsorption. Thus, if the theory is correct, the reabsorption of sodium and of bicarbonate should be mutually dependent. The theory has been challenged recently on the basis of micropuncture studies and is at present the subject of controversy. We found previously (Project ZO HL 01207-01 KE, 1974-1975) that sodium reabsorption was inhibited 33% by the absence of bicarbonate. The present studies were intended to test the converse, i.e. whether bicarbonate transport is inhibited by the absence of sodium and/or sodium transport, as predicted by the theory.

Methods

Proximal convoluted tubules were dissected from rabbit kidneys and perfused in vitro as previously described. A micro calorimetric method (developed by G. Vurek of the Laboratory of Technical Development, NHLI) was used to measure in nanoliter samples of tubule fluid the concentration of total CO_2 which is approximately equal to the concentration of bicarbonate at physiological pH.

Major findings

1. When tubules were perfused at slow rates in a bath containing 25 mM bicarbonate, the total CO_2 concentration in collected fluid was approximately the same whether the concentration of bicarbonate in the perfusate was zero or 25mM. Therefore, a steady state of bicarbonate concentration in the tubule lumen is approached under these conditions. The mean concentration was 9mM in the control state.

2. When sodium was eliminated from the bath and lumen and replaced by choline, tetramethyl ammonium, or lithium, the total CO_2 concentration in collected fluid at slow flow rates increased to 21mM, indicative of less effective bicarbonate transport. Therefore, bicarbonate transport is partially, but not completely, dependent on sodium. (If the dependence were complete, the concentration of total CO_2 would approach 25mM, which is the level in the bath upon elimination of sodium.)

3. The absolute rate of absorption of total CO_2 (measured at faster perfusion rates) was 15 pM cm^{-1} sec^{-1}. CO_2 absorption was greatly reduced when sodium was eliminated.

4. Inhibition of sodium transport by eliminating K from the bath or adding ouabain to the bath had the same inhibitory effect on bicarbonate transport as did eliminating sodium. Therefore, transport of sodium and of bicarbonate are mutually dependdent, as predicted by the theory discussed earlier.

5. The leak of total CO_2 from bath to lumen was measured by perfusing the tubules at a rapid rate with bicarbonate-free solutions while 25 mM of bicarbonate at pH 7.4 was in the bath. The leak was too fast to quantify accurately, suggesting that a large fraction of the total CO_2 permeates as dissolved molecular CO_2 rather than as bicarbonate (which has a much lower permeability).

Significance to biomedical research

Excretion of bicarbonate and sodium are major regulatory functions of the kidney, which remain incompletely understood despite considerable previous experimental effort. The present studies afford direct evaluation of the pertinent variables and represent an important step in elucidating the problem.

Proposed course

To develop a pH sensitive electrode in order to quantify other aspects of urinary acidification.

Publications

Cardinal J., Lutz, M., Burg, M., and Orloff, J.; Lack of relationship of potential difference to fluid absorption in the proximal renal tubule, Kid. Intl., V. 7:94-102, 1975.

Burg, M. : The mechanism of fluid absorption by proximal convoluted tubules, Proc. of the VI Intl. Cong. of Nephrology, June 1975.

Boudry, J.F., Stoner, L. C., and Burg, M. D. : The effect of acid lumen pH on potassium transport in renal cortical collecting tubules, Am. J. Physiol, V. 230:1, pp. 239-244, Jan 1976.

Burg, M. D.: Tubular chloride transport, Kid. Intl., V. 9, No. 2, pp. 189-197, 1976.

Burg, M. & Stoner, L.: Renal tubular chloride transport, Am. Rev. of Physiol., in process.

Burg, M.: Renal tubular chloride transport & diuretics, Circulation, in process.

Burg, M., Patlak, C., Green, N. and Villey, D.: The role of organic solutes in fluid absorption by renal convoluted tubules, Am. J. Physiol, in process.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01211-02 KE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Anion transport across individual amphibian erythrocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Floyd M. Kregenow, M.D. Staff Member KE NHLI

OTHER: Larry C. Stoner, Ph. D. Dept. of Physiology
Medical School
Syracuse University

COOPERATING UNITS (if any)
Dept. of Physiology, Medical School, Syracuse University
Syracuse, New York

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The electrical and transport properties were studied across a single red blood cell from the salamander, Amphiuma means.

PHS-6040
(12-75)

Objectives

Last year we developed a system for measuring electrical and transport properties across giant red cell from the salamander, Amphiuma means. We chose to study anion transport as a way of testing the system. A collaborative effort, using the single cell system has continued this year with Dr. Larry Stoner of Syracuse University.

Methods

Individual amphibian red blood cells are held snugly in a constriction in a specially prepared glass pipet. In this position the cell is a cylinder with hemispherical ends. A second glass pipet is centered within the pipet that holds the cell. Either of two types of inner pipettes is used. 1) a glass microelectrode ("Ling-Gerard" type) which punctures the cell membrane for measurement of cellular voltage and resistance, or 2) a larger pipet which does not enter the red cell but is used to superfuse one end of the cell with a radioisotope containing solution. Permeability to the isotope is determined from the amount of radioactivity that penetrates the cell and appears in the external bath.

Major Findings

1) To test the viability of single Amphiuma red cells in the holding capillary, we measured the membrane potential under experimental conditions designed to vary both the intracellular voltage and the chloride ratio across the membrane. It is generally accepted that in the red cell, the intracellular voltage results from a Gibbs-Donnan equilibrium, determined by the amount of fixed negative charge inside the cell. Cl is also believed to be distributed passively; consequently, the Cl concentration ratio, inside to outside, serves to measure the membrane potential. Thus altering the difference in the amount of fixed negative charge inside the cell relative to outside, should produce predictable changes in both the membrane potential and Cl ratio.

We varied the relative quantity of fixed charge within the cell in two ways. In the first method the absolute quantity of fixed charge within the cell was changed by taking advantage of the fact that most of the fixed charge is associated with the hemoglobin molecule and is pH dependent.

At physiological pH, the cytoplasm is more alkaline than the hemoglobin isoelectric point (the pH at which hemoglobin has no net change). Reducing the pH of the medium reduces the pH of the cytoplasm, and brings it closer to the isoelectric point. This, in turn, reduces the quantity of fixed negative change. In the second method we kept the total quantity of fixed charge within the cell constant, albeit low, by bathing the cells in an acid medium, and produced a further reduction in the concentration difference for fixed charge by replacing some of the extracellular Cl with an impermeant anion, PAH. The impermeant anion acted as an externally-placed fixed charge and produced a relative reduction (inside minus outside) across the membrane. This approach made use of a heretofore unknown quality of net Cl transport; i.e., an acid pH dramatically inhibits the net Cl movement out of the cell; presumably because this pH reduces Cl-OH or Cl-HCO_3 exchange across the membrane. It permits one to lower $[Cl]_o$ (for replacement with PAH) without lowering $[Cl]_c$.

The results of both approaches indicate that there is a direct relationship between the intracellular voltage and the negative logarithm of the Cl ratio. Thus, the study suggests that aspiration of the cell into the holding capillary has not damaged the cell. It also represents the most direct study to date of the factors determining the red blood cell potential.

2) To provide additional evidence that the ^{36}Cl flux across this preparation, (see last year's annual report) is through cell membranes rather than around the cell, we examined two agents which are known to inhibit ^{36}Cl flux in human red cells. The first agent is a disulfonic stilbene derivative, called SITS. It is a potent inhibitor of anion transport and has been used extensively by Rothstein and colleagues to characterize a membrane protein involved in anion transport. In the 10 cells we tested SITS ($5X10^{-4}M$) reduced the flux by 93±9% (mean±S.E.). The second agent, furosemide, is a less potent inhibitor of ^{36}Cl movement, but has added interest because at the concentration we used in this study, 1mM, it completely inhibits the process of "cell enlargement." (See accompanying annual report.) Furosemide inhibited the ^{36}Cl flux across the single Amphiuma red cell by 30%.

Significance

The measurements collected to date, provide some of the most direct observations available on how a cell handles chloride. In theory one could apply the technique to the study of other permeant molecular species and to other biological problems requiring single cell measurements.

Proposed Course

The studies using the single cell technique continue. We are currently examining how variations in $[Cl]_o$ and $[Cl]_c$ effect the ^{36}Cl flux. We will use the system to further study the "volume controlling mechanism" in the future.

Publications

Stoner, L. C., and Kregenow, F. M.: Chloride fluxes and voltage measurements in single red blood cells. Biophys. J. Abstracts 20th Annual Meeting, p. 170a, Seattle, Wash. February 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01212-02 KE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Volume regulation in nucleated erythrocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Floyd M. Kregenow, M.D.	Staff Member	KE	NHLI
OTHER:	Betty Nordan	Chemist	KE	NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.9	.5	.4

SUMMARY OF WORK (200 words or less - underline keywords)

This study examines that aspect of the "Volume Controlling Mechanism" responsible for cell enlargement. Duck red cells were introduced into hypertonic solutions and the effects of varying the $[Na]_o$ and $[K]_o$ examined.

PHS-6040
(12-75)

Objectives

Studies from this laboratory, using avian erythrocytes as a model, originally identified and then characterized a transport mechanism capable of regulating cell size. This "volume controlling mechanism" returns cells to their original isotonic volume in either isotonic or anisotonic media. It functions by controlling the movement of K into and out of the cell; the K, in turn, is accompanied by diffusable anion, primarily Cl, and osmotically obliged water. Changes in size result from shift in cell water. The primary process, the controlled movement of cation, occurs through membrane pathways which had been categorized previously as "leak" (in contrast to "pump") pathways. Two different pathways function depending upon whether the cells are in an enlarged or shrunken condition and thus need to either lose (cell shrinkage) or gain (cell enlargement) cations.

As reported previously, during cell shrinkage, the mechanism monitors K loss, in response to changes in cell size, by altering the resistance of a K pathway to K movement. Studies using cells with differing electrolyte content indicate that the cation pathway is relatively specific for K but that the anion pathway lacks specificity. The driving force for the K movement is simply the K electrochemical gradient.

On the other hand the process of cell enlargement is not as readily explicable. For instance, cells enlarge not only in hypertonic media, consequent to the initial osmotic shrinkage, but also in isotonic media in response to catecholamines. In response to either stimuli, both Na and K move rapidly in and out of the cells. When the external concentration of K, is 2.5mM, these cation movements do not produce a change in the Na and K content of cells or their volume. At slightly higher $[K]_o$, however, the cells accumulate the Na and K salts necessary for enlargement, and swell. Cells gain cations because of an imbalance in these rapid bidirectional fluxes; influx exceeds efflux. The newly acquired Na is then extruded by the cation pump in exchange for extracellular K. The final result is an enlarged cell, containing more K, Cl and water. It should be emphasized that the newly acquired K enters through two separate mechanisms, the increased net flux associated with volume regulation and the classical Na-K exchange pump. Only the latter is inhibited by ouabain.

Two aspects of cell enlargement require elucidation: 1) the nature of the transport process that causes the rapid bidirectional cation fluxes and 2) the reason that net cation accumulation only occurs when the $[K]_o$ exceeds 2.5mM.

Earlier studies (1973-74) provided a limited understanding of the first of these two problems. These experiments suggested we were dealing with a co-transport process, if one restricted the analysis to external K concentrations of 2.5mM or less. In brief, the following experiments supported this model. First, the enhanced Na influx, K influx and K efflux showed an absolute dependence for the presence of both external Na and K. Second, these same three fluxes gave a curvilinear response when studied as a function of $[Na]_o$ and $[K]_o$ that approximated Michaelis-Menton kinetics and third, at a $[K]_o$ of 2.5mM, the K electrochemical potential was equal (+96mv) and opposite to the Na electrochemical potential (-94mv). Thus at this $[K]_o$, there were adequate driving forces, giving the postulated mechanism, for the rapid cation movements in both directions, and an adequate explanation, since there was no net force, for the absence of any net cation flux.

All these findings were compatible with a model in which a mobile carrier translocated Na and K across the membrane only when combined with both cations. If the carrier combined with only one cation, it could not cross the membrane. In this model, Na entry into the cells was driven by its electrochemical gradient and K was dragged against its gradient. Similarly, the driving force for K exit from the cells is the K electrochemical gradient and Na is carried out of the cell against its gradient.

On the other hand, there were several observations, that were difficult to explain by the model. For instance, Na efflux did not decrease when extracellular K was removed despite the observed decrease in K efflux. Under these conditions, the enhanced Na efflux was evidently not coupled to K efflux.

Thus, although the co-transport model has been helpful, it has proved difficult to conceive of a simple version which encompasses all of the findings. A more thorough understanding of the process of cell enlargement remains one of our major objectives.

Major Findings

1) As noted above the increase in Na and K fluxes in response to norepinephrine had been previously characterized in detail. Having made this analysis when norepinephrine was the stimulatory agent and having established previously that the transport process was similar upon addition of

cells to hypertonic media, we chose to examine and model the response using hypertonic solutions. With hypertonicity as the stimulus three of the four fluxes that constitute the rapid bidirectional cation movement, K influx, K efflux, and Na influx, varied directly with the extracellular Na or K concentration. The curves which describe the relationship between these three fluxes and $[Na]_o$ or $[K]_o$, fit, to a first approximation, Michaelis-Menton kinetics and approach zero as either $[Na]_o$ or $[K]_o$ approach zero. On the other hand, the other flux component, Na efflux, was unresponsive to changes in $[Na]_o$ or $[K]_o$.

Thus, the results of the study are identical to those with norepinephrine. A model designed to explain the findings, is similar to the one described above. It also has identical short comings. Expecially noteworthy, is the fact that of the 12 curves generated by varying the $[Na]_o$ and $[K]_o$, all were qualitatively and quantitatively similar to their norepinephrine counterpart. We infer that the effector in the response initiated by norepinephrine is identical to that initiated by hypertonicity.

2) In a continuing effort to test whether the pathways related to cell shrinkage are spatially separate from these associated with cell enlargement, we focused on three experimental conditions which differentially effect the phenomena. We chose to examine the component of transport which is similar in both phenomena, namely the increase in K efflux.

Low $[K]_o$ (< .1mM) has no effect on the K efflux component associated with cell shrinkage, but inhibits by more than 80% the component associated with cell enlargement. 1 M furosemide inhibits the K efflux component of cell shrinkage by 15% but completely eliminates the component associated with cell enlargement. Finally, replacing both the extra and intracellular Cl with SO_4 inhibits the K efflux component of cell shrinkage by 66% but is more than 97% inhibitory to the component associated with cell enlargement.

We infer from these studies the pathways are most likely spatially separate. More conclusive evidence would be the discovery of an agent which inhibited the flux component associate with cell shrinkage without affecting the component related to cell enlargement.

Significance

The importance of the "volume controlling mechanism" stems from the fact that it represents transport processes quite distinct from the classical cation pump, yet it is composed of cellular elements which impart to it a degree of precision and variability as unique as one finds with the pump. It is also of broad biological importance because the mechanism allows cells to control their volume dynamically and as a corollary to develop fragile and malleable cell surfaces - properties essential to most animals. It is likely that this novel "volume controlling mechanism" is implicated in a number of biological phenomena both physiological and pathological which remain to be worked out in detail.

Proposed Course of Project

Studies aimed at a more thorough understanding of the process of cell enlargement are in progress.

Publications

Kregenow, F. M., Robbie, D. E., and Orloff, J.: Effect of norepinephrine and hypertonicity on K efflus and cyclic AMP in duck red cells. Am. J. Physiol. June 1976.

Kregenow, F. M.: In book "Membrane transport in red cells." Chapter entitled "Transport in avian red cells." Edited by Clive Ellory, Academic Press Inc. (London) limited in Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01216-01 KE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Pathways of chloride movement across Necturus proximal tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Kenneth R. Spring, DMD, PhD. Sr. Staff Fellow KE NHLI

OTHER: None

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2		

SUMMARY OF WORK (200 words or less - underline keywords)

The pathways and mechanisms of transepithelial transport of chloride ions are being investigated in the proximal tubule of the Necturus kidney. Chloride sensitive intracellular microelectrodes were constructed to monitor intracellular chloride activity and to determine cellular responsiveness to changes in extracellular chloride concentration.

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PHS-6040
(12-75)

Project Description

Objectives

No definitive information is available concerning the mechanism (s) by which chloride ions move out of the lumen of the renal proximal tubules of the amphibian, Necturus maculosus. Two possible pathways must be considered: 1) chloride ions may diffuse across the spaces between the tubular epithelial cells; 2) chloride may enter the tubule cells crossing the luminal cell membrane alone or in conjunction with a cation(e.g. sodium). Distinguishing between these two possibilities is essential to understanding the mechanism of transepithelial transport of salt and water. Since each of the two modes of transepithelial chloride transport has distinctly different consequences for models of tubular electrolyte reabsorption, they will be discussed in further detail. 1) Extracellular chloride movement requires favorable electrochemical gradients for that ion across the intercellular spaces. Since the lumen of the Necturus proximal tubule is 10 to 15mv negative compared to the interstitium, an electrical driving force exists across the epithelium favoring chloride reabsorption. Chemical measurements of luminal chloride indicate a transepithelial distribution in keeping with passive ion movement and a chloride permeability sufficiently high to achieve the observed fluxes. In the only published attempt to evaluate the tracer movement of chloride the conclusion was reached that passive movements could account for a portion of the observed fluxes. Considerable "exchange diffusion" was also postulated to occur, but the experimental approaches used were not adequate to confirm this assumption. 2) It was calculated previously that chloride ions could not be passively distributed across the luminal membrane of the cells of the Necturus proximal tubule. The intracellular chloride activity would have to be below 2mM to enable such a passive distribution. This conclusion was based on the observation that the cell interior is approximately 50mV negative to the tubule lumen thereby constituting an unfavorable environment for anion entry into the cell interior. The only experiments available concerning this point demonstrate that the intracellular chloride activity is 18mM/kg cell H_2O, far in excess of the level consistent with a passive distribution. Experiments in other epithelia have demonstrated coupled entry of Na and Cl across the comparable membranes of small intestine, toad urinary bladder and frog skin. Such neutral entry can occur in spite of unfavorable electrochemical gradients for one of the ions. Another possibility is an anion exchange across the luminal membrane involving chloride and bicarbonate ions. Since such an exchange is electrically neutral, it too could occur in the presence of an unfavorably electrical gradient.

The principal object of this investigation is the quantitation of the fluxes of chloride ions across the cellular and extracellular pathways of Necturus proximal tubules. In addition estimates for cellular membrane chloride permeability as well as extracellular permeability will be obtained. Finally, direct information on the potential dependence of the movement of chloride ions will be obtained.

Methods:

Microelectrodes which sense chloride activity and are small enough to be inserted into tubule cells have been developed. Glass electrodes are fabricated utilizing a microfiber glass capillary specially drawn in the NIH glass shop. Two capillaries each 1 mm in outside diameter, are heated and twisted together in a microelectrode puller. They are then drawn to a tip of less than 1 μm. One barrel is exposed to the vapors of a volatile siliconizing agent (dimethyl-dichlorosilane) for 60 seconds and the electrode baked for 1 hour at 100°C. After cooling, the siliconized barrel is partially filled with chloride ion exchange resin and the other barrel filled with 3M KCl for use as a voltage sensor. Since the chloride sensing barrel has a very high electrical resistance (10^{11} ohms) it exhibits slow response time and great sensitivity to electrical interferences. It is connected to a high impedance electrometer whose output is displayed on a digital voltmeter, interfaced to a small computer. Determination of the end point of electrode drift and slope sensitivity to chloride is then made objectively by this computer system.

The following experiments will be performed on kidneys perfused with artificial solutions. The methods for kidney perfusion are well established and have previously been utilized by the principal investigator. Briefly, the aorta, vena cava and caudal veins are cannulated. The aortic perfusate supplies the glomerular capillaries and the caudal vein supplies all the other capillaries in the kidney. Such an arrangement allows precise control over the composition of the extracellular fluid. Luminal fluid may be changed by the insertion of micropipettes using standard micropuncture methods. Luminal chloride concentration may be rapidly varied after a chloride sensing electrode has been placed in a tubule cell in the perfused segment. If the intracellular chloride activity alters in response to variations in luminal chloride concentration, additional experiments will be performed. Measurements of fluid reabsorption, electrical potentials and intracellular chloride activity will enable calculation of cellular chloride permeability and transcellular fluxes. Variation of luminal membrane potential may be accomplished by the voltage clamp method, and the effect of changes in potential on chloride activity determined. Information about the potential sensitivity of the chloride flux may be used to determine the extent of neutral or exchange entry.

Major Findings

Since this project began in January, 1976 the results to date concern only the behavior of the ion sensitive microelectrodes. Electrodes are now routinely constructed with slope sensitivity of 50mV per decade change in chloride concentration. They exhibit little sensitivity to variations in cation composition and good selectivity for chloride over bicarbonate or phosphate. The feasability of impaling tubule cells with double-barrel microelectrodes was investigated and no significant difficulties observed.

Proposed course of project

Based on the results from intracellular activity measurements one of two courses will be chosen. Either continued study of variations in intracellular chloride activity as a function of chemical and electrical driving forces, or investigations into the chloride permeability properties of the extracellular pathways. In either case the methods to be utilized will include the voltage clamp and tracer flux methods developed previously by this investigator.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01217-01 KE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanism of epithelial fluid transport, the interspace volume clamp

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Kenneth R. Spring, DMD, PhD. Sr. Staff Fellow, KE NHLI

OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2		

SUMMARY OF WORK (200 words or less - underline keywords)

An optical-electrical system has been developed to enable precise, quantitative control of the dimensions of the intercellular spaces of flat epithelia. Light absorption due to dye-filled spaces between cells is used as the input to a voltage clamp circuit which passes transepithelial electric current. The electric current response to various physical forces applied across the tissue may be used to develop more realistic models of epithelial fluid transport.

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PHS-6040
(12-75)

Objectives

The primary goal of this investigation is the generation of information about the role of changes in the geometry of intercellular spaces in the regulation of epithelial fluid transport. The methods employed represent a unique blend of optical and electrical techniques developed specifically for this investigation.

The observations that leaky intercellular shunts are present in many epithelia has led to extensive investigation of this paracellular pathway. It is widely accepted that much of the passive movement of ions occurs via extracellular pathways in such tissues as renal proximal tubules, gall bladder and intestine. Many investigations have centered on the extent to which ions bypass the cells and how this shunting determines the functional state of the epithelium. Variations have been observed in the tightness of the "tight junctions" which ring the mucosal surface of epithelial cells together with alterations in the width and length of the spaces between the cells. All experimetal approaches to date fall into two categories: 1) An experimental variable is manipulated, and the dimensions of the intercellular spaces and/or junctions are subsequently determined by electron microscopy. 2) A maneuver is undertaken which is thought on the basis of previous evidence (e.g. case 1 above) to alter the properties of the extracellular pathway, and various physiological variables are monitored. Both of these approaches suffer serious limitations. Electron microscopy is inadequate to resolve changes in junctional morphology and conflicting reports have resulted depending on fixation procedures. The time constants for relaxation of the morphological changes may be short enough to preclude accurate microscopy even if rapid fixation is employed. Minor changes in the paracellular pathway lead to very dramatic changes in a number of functional parameters. For example, intercellular space widening due to increased hydrostatic pressure on the serosal (blood) side of an epithelium leads to increased electrical conductance, solute permeability, and hydraulic permeability, decreased net fluid transport, lower electrical potential difference and alterations in ion selectivity patterns. So many parameters change it is difficult to interpret the results and causal relationships cannot be definitively established. It is apparent that what is needed is an on line, real-time system for monitoring functional parameters and tissue morphology simultaneously. Further, it would be most desirable to control the tissue morphology, as well.

A system has been developed for this purpose and may be described as an "interspace volume clamp." The principle upon which this experiment operates is the following: the output of an optical monitor of interspace dimensions is used as the input to a control circuit which maintains the interspace dimension at a con-

stant value. The control circuit consists of a current passing system of operational amplifiers which alter interspace dimension by varying the amount of transepithelial current flow. Thus, an increase in serosal hydrostatic pressure, which would ordinarily lead to a widening of the intercellular spaces, may be opposed by electric current flow which would ordinarily narrow the spaces. Since no change in interspace dimension occurs the effect of pressure alone on epithelial parameters may now be observed. In addition the current required to oppose the pressure may be used in model equations to determine other system parameters. Interplay of various physical forces in this way leads to a series of relations describing tissue responses in the absence of morphological changes.

Methods:

The tissue of choice for these experiments in the gall bladder of the amphibian Necturus maculosus. The cells are 25 to 30 μm in diameter and 40 to 60 μm tall. The intercellular spaces are readily visualized and can be observed to open and close in response to hydrostatic or electric forces. This tissue is mounted in a specially designed chamber modified from a Dvorak-Stotler tissue culture chamber. The tissue is sandwiched between two mica plates so that approximately 0.75mm^2 is exposed to mucosal and serosal bathing solutions. Fluid constantly perfuses both mucosal and serosal baths. Electrical bridges enable potential measurements and passage of electric current. The total thickness of the chamber is 1.2mm and it so designed to fit within the optical path of a high power light microscope.

The chamber is placed on the stage of a Leitz inverted microscope equipped with brightfield optics. The condenser is replaced by an inverted objective lens to improve the focus and sharpness of the image. The objective lenses are standard high power dry and oil immersion. The viewing head is equipped with a beam splitter which is used to divert light from the image to a photomultiplier tube. The current output from the photomultiplier tube is used to indicate the size of the intercellular spaces. A wedge monochrometer is placed in the path of illumination and the tissue is exposed to a solution containing a high molecular weight dye. This dye fills the extracellular spaces but does not penetrate through the tight junction. Adjustment of the slit of the photomultiplier enables measurements to be made from one intercellular space. The density of light absorption by the space is proportional to the dimensions of the space thereby providing a quantitative measure of change in morphology. The output of the

photomultiplier is used as the input signal to a conventional voltage clamp circuit thereby becoming the controlled variable. Eventually the various signals will be digitized and analyzed by a small computer.

Major Findings

Development of the chamber and optical systems is complete. Living gallbladder placed in this arrangement may be clearly visualized and maintained in a viable state. A number of dyes have been tried and several appear promising. Evaluation of the system stability and resolution are presently underway.

Proposed Course

In addition to the data about the geometrical effects of physical forces on epithelial properties, information should be obtained on many of the physical properties of the tissue. Static pressure-volume curves of the cell membranes, electrical potential differences and pH gradients may all be discerned by the use of the appropriate indicator dyes. With the advent of potential dependent dyes it is hoped that information on the potential gradients along intercellular spaces will also be obtained.

Finally, further development of models for fluid absorption by epithelial await the specific data which should be provided by these experiments. The versatility of the system makes it feasible to utilize other flat sheet epithelia in similar experiments.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART AND LUNG INSTITUTE
JULY 1, 1975 to JUNE 30, 1976

This laboratory develops instruments and methods applicable to biomedical research, diagnosis or therapy relevant to the mission of the institute. This group of scientists having an awareness of the limitations of current methods and instruments anticipates how new instrumentation incorporating advances in physical science and technology can be developed to facilitate biomedical research.

The development of new methods and instruments for biomedical research in a new and highly specialized field is generally shunned by industry because of the inability to predict development costs, anticipate the market, or the return on the investment.

We develop working systems that can be applied at N.I.H. in conjunction with ongoing research activities and make the results available to the scientific community and industry by publishing the method or instrument. The areas of our activity include analytical methods and separation techniques, not only for chemicals but also for living cells; clinical applications includes materials and methods development for better cardiopulmonary support for both isolated organs and whole animals.

The Section on Pulmonary and Cardiac Assist Devices continues to improve extracorporeal gas exchange systems and evaluate their research and clinical potentials.

An improved hypothrombogenic membrane lung has been developed and tested in lambs. No clotting was observed in extracorporeal membrane lung circuits maintained for several days without anticoagulant. In addition, further improvements have eliminated the reduction in cells and platelet counts that has heretofore always accompanied extracorporeal circulation of blood.

The hypothrombogenic surface was evolved through a program of identification of surface defects created by submicroscopic projections of silica reinforcing material and impurities used to produce silicone rubber of sufficient strength. The composition of the silicone membrane material, fillers, curing agents, and processing techniques were examined in detail by testing in a complete working extracorporeal system in chronic experiments using lambs to evaluate minute changes as an index of merit. A composite membrane of pure gum silicone radiation cured, a carbon filled layer, and a silica strengthened layer has been selected and demonstrated in chronic extracorporeal systems to cause no clotting or changes in the number of cells or platelets. The findings have been reported for lambs but not applied to clinical support in humans.

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The possibility of long term gas exchange without excessive cell or platelet damage and threat of hemmorrhage has led to the consideration of additional therapeutic advantages of extracorporeal gas exchange. The ability to clear all the carbon dioxide produced by the body using a low flow extracorporeal gas exchange system is being studied.

Several investigations are concerned with the development of ultramicro and smaller samples to permit repetitive measurements of blood gasses, electrolytes and enzymes to evaluate their application to research methods where the samples are limited by the technical demand or for clinical applications where repetitive measurements and the patient compete for blood volume.

A microelectrode for oxygen eliminates delay and spurious responses by reducing the permeable idea of membrane to the pores of a porous silver mesh electrode applied without electrolyte to the cathode. The porous area creates a tight rate-of-diffusion barrier and high sensitivity measuring circuits record the minute reducing current. The electrode is expected to have rapid response, minimal oxygen consumption, and freedom from pressure and motion artifacts, and be applicable to thin surface films where the low consumption would be required to measure without significant oxygen consumption.

The transcutaneous measurement of PO_2 has been studied with similar electrodes evaluating electrode design and various methods of maintaining skin perfusion.

A system for measurement of PO_2 and PCO_2 in extracorporeal circuits for more than 24 hour periods without blood loss has been further improved, tested, and the results published. Oxygen is analyzed by amperometry and CO_2 by calorimetry of the reaction of carbon dioxide with lithium hydroxide. Tests have shown the system to be stable within 5% for 24 hour periods.

The basic simplicity of ion-specific electrodes makes them attractive for the measurement of certain electrolytes in automated blood analysis instrumentation. Steele, et. al (Proc. San Diego Biomed. Sympos. 14: 469-478, 1975) have used a programmable calculator system to overcome many of the well-known problems of ion-specific electrodes - interferences, drifts, and liquid junction potential changes. They have also shown that such a calculator system makes it possible to quickly convert the activities which ion-specific electrodes measure into the concentration units which are widely employed clinically for serum electrolytes. This conversion, is based on factors (serum water content, activity coefficients and ion binding by proteins) which may vary significantly in critically ill patients. In order to further establish the advantages and limitations of ion-specific electrodes for the determination of serum electrolytes of critically ill patients, a programmable calculator and data acquisition system with capabilities greater than that of Steele, et al has been assembled, programmed and put into operation. The construction of a pH electrode and the design of a total CO_2 measurement device have been undertaken with the objectives of great simplicity and small sample size.

The Italian - U. S. Science Cooperative Project has thoroughly tested the oxygen dissociation curve apparatus designed for both clinical and research use. Particular attention has been paid to the problems of determining the saturation from 0-2% and 98-100% to ±2%. The rest of the curve being readily determined to this precision. A large number of normal and abnormal hemoglobin patients have been tested. Some automation of the system has been achieved through the use of a microprocessor and a totally new instrument which will be completely automated is under construction. Work has started on the laser flash photolysis system for whole blood work. A novel optical method for very accurately obtaining the concentration of the various hemoglobin derivatives is also under construction.

Laser light scattered from tissues in-vivo is broadened in linewidth due to the doppler shift produced by moving red cells in the microcirculation. A feasibility study was carried out to demonstrate the use of this effect to measure and monitor tissue blood flow. Light from a helium-neon laser illuminates a 1 mm area of tissue (human skin or rat renal cortex) and the back scattered light is detected with a photomultiplier. The spectrum of the doppler beat notes is analyzed directly with a digital spectrum analyzer, or processed by analog circuitry to yield a flow parameter based on the root-mean-square linewidth. This parameter was compared with ^{133}Xe washout in the skin of 7 volunteers subjected to varying degrees of ultraviolet-induced erythema, and found to vary in an approximately linear manner with skin blood flow. The laser instrument provided continuous monitoring of the blood flow fluctuations, including the pulsatile component. The instrument was used to monitor flow in the outer cortex of the rat kidney during transient and steady state administration of norepinephrine, angiotensin, hydralazine, dextran, dopamine, nitroprusside and angiotensin blocked by saralasin. The dynamic and steady state responses were consistent with known parmacology and renal physiology, and with the assumption that vasoconstrictor angiotensin II receptors in the kidney are accessible to blood-borne inhibitors. The laser doppler method appears to be a promising tool for rapid monitoring of tissue perfusion.

The nuclear magnetic resonance method for evaluation of blood flow has been demonstrated to be applicable to measurement in humans in our contract supported program at Medical College of Wisconsin. Flow measurements have been made in limbs and within the head entirely by harmless electromagnetic fields utilizing electronic techniques developed here. The results support the idea that we can use this method to evaluate regional blood flow in the head and elsewhere non-invasively.

Development of the batch microcalorimeters has been continued with major emphasis being placed on improvement of sample cells, electronics temperature regulators and the development of a microprocessor for data processing. Several enzyme reactions are being studied with a view to clinical analysis. Because of the large amounts of enzymes needed for the required sensitivity several of the enzymes are being immobilized on glass beads so that they can

be used repeatedly. A fluidic logic flow system has been developed and tested on the stopped-flow microcalorimeter. Both this unit and the syringe drive unit are operating well and the only remaining problem is obtaining rapid, thermoequilibrium of the incoming solutions. The artifact from mixing is less than 1 microcalorie. A new combined pH-thermal titration cell has been constructed and tested. Several improvements are being made and a differential cell is also under construction. A sensitivity of 0.1 milli-degree is now routine in a 2 ml sample.

A 3 millisecond response time thermistor has been incorporated into a stopped-flow apparatus. It will be used in the study of reaction of carbon dioxide and phosphates with hemoglobin at various stages of oxygenation. Lycra coatings for pH electrodes have proved very effective in protecting electrodes from the effects of protein. This new electrode will be introduced into both stop flow titration and PCO_2 electrode systems. Difficult problems associated with the manufacturer of observation tubes for the high speed optical flow apparatus and clean signals with a consistent time resolution of 200 microseconds are presently being obtained. The kinetics of the reaction of calcium and EGTA at 25°C and 4°C over the pH range of 6.5-7.5 will be continued on this instrument. A laser flash photolysis apparatus for studying the very fast reactions of oxygen in both hemoglobin solutions and red cell solutions has been initiated and that the laser has been set up and operated. The optical system and cell for hemoglobin studies constructed and installed.

Studies of the interaction of proteins with fluorescent ligands used as probes for macromolecular structures includes: 1. Binding studies on the interaction of cationic fluorescent dyes with alcohol dehydrogenases and serum albumins. Such dyes appear to bind in areas distinct from those occupied by more commonly used anionic fluorescent probes. 2. Spectral characterization of quinacrine mustard conjugates of proteins in order to establish the usefulness of this dye in protein chemistry as well as nucleic acid and chromosome studies. 3. Preparation and characterization of protein conjugates containing a new dye, N-105, made by Walter Steward of NIAMDD. The dye is designed for fluorescence microscopic localization of nerve processes following microinjection into the axoplasm of individual nerves.

Novel applications of fluorescence spectroscopy in problems of biochemical interest include: 1. The study of the binding of the fluorescent antibiotic pimaricin to red cell membranes by stopped-flow fluorescence. Since pimaricin fluorescence enhancement is thought to occur upon binding to membrane cholesterol, the biphasic kinetic plots suggest the presence of two types of cholesterol pools. 2. The rate of labeling of proteins by fluorescent dyes was found to be measurable by stopped-flow fluorescence. Conjugates made by reaction with fluorescamine and quinacrine mustard have higher fluorescence yeild than the free dyes. The rates of reaction are altered by competing ligands and modifications of protein structure.

A new foam separation technique has been developed using the flow-through coil planet centrifuge. Under a rotating centrifugal force field, foams are created within a long fine coiled tube to carry foam active materials through the solvent trapped in each turn of the coil. Rhodamine B introduced at 1 ppm was almost entirely recovered at 620 ppm. High-speed chromatographic sepaation is possible with a small amount of sample.

A simple and accurate micromethod for the determination of cell osmotic fragility is introduced. The method uses the Poiseuille's parabolic flow pattern together with the gravity to retain cells in a long capillary tube while a solution with decreasing osmolarity is passed through the tube for hemolysis. Hemoglobin released from the cells is quickly removed by the axial flow pattern and monitored with a spectrophotometer for recording the hemolysis curve. A comparison is made with present methods and test results from normal and abnormal individuals are reported. Advantages are simplicity, reproducibility, and accuracy with 1 to 2 µl sample doses and versatility for possible automation in clinical laboratories as well as a research tool for various types of cell studies.

The heat evolved from individual cells in a microscopic field modifies the thickness of a condensing film of oil on a thin supporting membrane to allow estimation of heat evolution from individual cells. Granulocyte ingestion of bacteria correlated with increased metabolic heat. Improvement in instrumentation to permit observation by transmitted light and application to specific cell measurements is projected.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01401-03 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Foam Countercurrent Chromatography

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Ito	Visiting Scientist	LTD NHLI
OTHER:	R. L. Bowman	Chief, Lab. Techn. Dev.	LTD NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

A new foam separation technique has been developed using the flow-through coil planet centrifuge. Under a rotating centrifugal force field, foams are created within a long fine coiled tube to carry foam active materials through the solvent trapped in each turn of the coil. Rhodamine B introduced at 1 ppm was almost entirely recovered at 620 ppm. High-speed chromatographic separation is possible with a small amount of sample.

PHS-6040
(12-75)

Project Description:

Objectives: Development of a new foam separation technique using the flow-through coil planet centrifuge.

Method Employed and Major Findings:

A. Principle: The principle of the method is demonstrated by a simple model which consists of a coiled tube rotating about its horizontal axis. Liquid and gas confined in the coiled tube soon reach a dynamic equilibrium state where each turn of the coil is occupied by a nearly equal amount of each phase starting from one end of the coil called "the head" and any excess of either phase occupies the other end of the coil called "the tail", as in the case of two-phase liquid systems. This dynamic equilibrium state forces a third phase with a density greater than the gas phase and smaller than the liquid phase to move toward the tail end of the coil. Thus, when the liquid contains surfactant, foams created by rotation of the coil undergo a countercurrent flow toward the tail end of the coil, carrying any solute or particles having an affinity to the foam. This countercurrent process can be enhanced if the gas is constantly supplied through the head to the tail end of the coil.

In order to achieve an efficient separation with a long fine coiled tube, it is necessary to apply a centrifugal force which prevents a plug flow under a high countercurrent flow and also quickly eliminates excess liquid from the foams. The flow-through coil planet centrifuge was used to provide a rotating centrifugal force field with respect to the coiled tube. Since the apparatus avoids the use of rotating seals, a desired number of flow tubes can be used to move the liquid or gas phase in and out through the rotating column against a pressure of several hundred psi.

B. Elution Schemes: The separation column to be mounted on the coil planet centrifuge is prepared from a 2.5 mm i.d., 30 m long Teflon tubing coiled onto 6 plastic pipes with 1.3 cm o.d., making approximately 1000 turns. The column is equipped with 4 flow tubes, the liquid collection line and the gas feed line on the head side and the liquid feed line and foam collection line on the tail side.

After the apparatus reaches the desired revolutional speed, the solvent containing a surfactant is pumped through the liquid feed line while N_2 gas is fed through the gas feed line. When the foams begin to elute from the foam collection line, the needle valve on the liquid collection line is opened to elute the liquid phase at the desired rate, while the solvent is switched to the sample solution. The foam flow rate through the foam collection line is equal to the difference between the liquid feed rate and collection rate and can be adjusted by the needle valve or any resistance placed after the valve. Consequently, the sample solution is continuously separated into two streams, the stripped solution from the liquid collection

line and the enriched foams from the foam collection line.

When the objective is enrichment of the foam active substance, the technique is modified in such a way that the substance is first retained in the column, washed, and eluted in a concentrated state. In this case the liquid collection rate is adjusted equal to or slightly below the liquid feed rate so that the foams are not eluted through the foam collection line and the active material is retained in the column. After the sample feed is completed, the washing solution is introduced through the liquid feed line to wash the column contents until the eluate through the liquid collection line becomes clean. Then the needle valve on the liquid collection line is closed and the liquid feed rate is slowed down to efficiently collect the enriched foams through the foam collection line.

A modified elution scheme is used for chromatographic separation of small amount of sample. The column is equipped with two flow lines, the gas/liquid feed line at the head and the foam collection line at the tail. The column is first equilibrated with the gas/liquid phase at a predetermined revolutional speed and the sample solution is introduced through the feed line. Then the column is fed with a mixture of gas and liquid phases at a given ratio through the feed line while the foams are fractionated through the foam collection line.

C. Results: Efficiency of the first scheme was examined on removal and enrichment of rhodamine B using sodium lauryl sulfate as a collector. Five hundred milliliters of sample solution containing 1 ppm rhodamine B, 100 ppm sodium lauryl sulfate, and 0.01 M NaCl, were fed at a flow rate of 120 ml/hr while the apparatus was operated at 360 rpm with a 30 cm revolutional radius. The pressure gauge at the N_2 tank was set at 200 psi which gave a flow rate of 4 liters per minute at the outlet of the foam collection line. The eluate through the liquid collection line was sampled at 100 ml intervals for fluorescence analysis of the dye. No fluorescence activity was detected until 300 ml sampling period when the activity rose to 0.00006 ppm which continued steadily up to the end of the elution. Then the retained dye was eluted through the foam collection line at 2.4 ml/hr. The dye was almost entirely recovered in less than 1 ml at a concentration of 620 ppm.

Capability of the second scheme was demonstrated on chromatographic separation of rhodamine B and Evans blue using sodium lauryl sulfate as a collector of rhodamine B. A sample size of 0.5 ml containing each component at 250 ppm was eluted at 6 ml/hr of liquid flow rate and 3 liter/minute of gas flow under 270 rpm. The rhodamine B was eluted in 20 minutes with a sharp peak while Evans blue began to appear in 3 hours with a broad peak which continued up to the end of the experiment.

Significance to Biomedical Research and Program of the Institute:

Foam separation techniques have been increasingly used for separations of

a variety of substances ranging from small ions to micro - and macroscopic particles. Despite their great potential in selectivity and versatility, the method still remains relatively unpopular because of the unavailability of an efficient apparatus. The new efficient method described above may open a broad application to biomedical and biochemical research.

Proposed Course:

1. Refinement of the apparatus.

2. Application to biomedical and biochemical research.

Publications:

1. Ito, Y., and Bowman, R. L.: Foam Countercurrent Chromatography: New Foam Separation Technique with Flow-Through Coil Planet Centrifuge. Separation Science, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01403-03 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Eye Motion Measurement by Ultrasound

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. W. Noble	Electronics Engineer	LTD NHLI
	R. L. Bowman	Chief, Lab. Techn. Dev.	LTD NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

Deviant motion of the eye at the first several harmonics of the heart rate may be indicative of circulatory problems within the head and neck areas. Since an acoustic wave travelling in air is totally reflected by any liquid on solid surface. it is possible to interrogate the front surface of the eye by means of ultrasound. The phase delay between separate transmitting and receiving transducers is modulated by the eye motion. The delay is converted to amplitude modulation and recorded, resulting in a record of eye motion vs. time.

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Objectives: Deviant motion of the eye at the first several harmonics of the heart rate may be indicative of circulatory problems within the head and neck areas. There is some evidence that variation from normal motion may warn of impending stroke.

Major Findings: Since an acoustic wave travelling in air is totally reflected by any liquid or solid surface, it is possible to interrogate a small region of the front surface of the eye by means of ultrasound. The phase delay between separate transmitting and receiving tranducers is modulated by the eye motion. This delay is converted to amplitude modulation and recorded, resulting in a record of eye motion vs. time.

Various methods of mounting the transducers with respect to the eye of human subjects have been investigated in an effort to reduce artifacts due to extraneous motion of the subjects head. The associated electronics have been improved, incorporating crystal frequency control, narrow band receiving equipment, and heavy amplitude limiting. Cross-correlation equipment has been employed to compare the eye motion with the patients electrocardiogram to discriminate against signals which are asynchronous with the heart.

Significance to Biomedical Research and the Program of the Institute:

If deviant motions of the front surface of the eye are found to correlate with intracranial circulatory pathology, a non-invasive survey method could be developed.

Proposed Course: Interference problems produced by random motions of the head and eye with respect to the transducers are of such magnitude that even cross-correlation with the ECG produces only a barely recognizable signal at the heart rate. The present system is not considered promising.

An alternative motion sensor which is simpler and more adaptable mechanically is under consideration. The eye motion is sensed by change in electric capacitance between the eye and a small fixed metal plate. This capacitance change frequency modulates a miniature oscillator, the output of which may either be demodulated in the fashion of an FM receiver or used to provide an audible indication of motion. The strength of the required electric field may be so small that tissue damage would not be expected, whereas in the case of ultrasound there may be some question. The exact position being interrogated on the eye surface is much more easily controlled by shaping and positioning small capacitive probes.

A second and potentially more powerful technique under consideration makes use of the coherence properties of laser light. Interferograms or holograms produced by interference between light reflected from the eye surface and a reference light beam can be interpreted in terms of motion of many individual points on the eye surface. In particular this method is

adaptable to measuring relative motion of various positions on one eye with respect to corresponding positions on the other eye, or with respect to a reference such as a point on the forehead, etc.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01404-08 LTD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Membrane Lung Systems for Long Term Support

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	T. Kolobow	Chief, Section on Pulmonary & Cardiac Assist Devices	LTD NHLI
OTHER:	L. Gattinoni	Visiting Fellow	LTD NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3-3/4	1-3/4	2

SUMMARY OF WORK (200 words or less - underline keywords)

It is the goal of this project to develop improved membrane lung suitable for long term pulmonary and cardiopulmonary support. Current interest centers around 1. improved blood compatible polymers, 2. the effect of carbon fillers on blood compatibility of polymers, 3. development of a carbon dioxide membrane lung for large volume carbon dioxide elimination, and 4. applying selective carbon dioxide elimination techniques to specific clinical situations.

PHS-6040
(12-75)

Objectives:

1. We are demonstrating advantages and the safety of the membrane artificial lung for short term and long term use. Presently, widely used bubble oxygenator systems used in open heart surgery cannot be applied safely in excess of 4 to 6 hours, and it would be desirable to increase the margin of safety of these devices. Similarly, there is a great need for artificial lung systems capable of satisfactory performance for many weeks in patients requiring respiratory or cardiorespiratory assistance.

2. Blood compatibility of synthetic biopolymers is the most important problem preventing wide spread application of artificial internal organs. This problem is especially critical in the use of devices with a large surface area such as the artificial kidney, and the artificial lung. However, such devices with relatively small exposed surface areas as heart valves, prosthetic ventricules, synthetic arteries, arterio-venous fistulae and many others have an equally important claim on future advances in biomedical materials research.

Our main emphasis lies in developing membranes highly permeable to respiratory gases, and yet highly thromboresistant.

3. We are investigating the possible application of highly permeable membranes in artificial lungs to a clinical population in need of extracorporeal carbon dioxide elimination with or without combined oxygen enrichment.

This population group includes, among others, patients with chronic obstructive pulmonary diseases. It is hoped that transient restoration of carbon dioxide exchange to a physiological range may temporarily improve pulmonary ventilation by mechanism not as yet clearly understood.

It is not expected that chronic obstructive pulmonary disease can be cured. Rather, we visualize a treatment protocol not unlike nowadays seen in artificial kidney patients, i.e., once or twice a week to once biweekly or thereabout.

Similarly, the use of extracorporeal carbon dioxide exchange devices may permit adequate pulmonary ventilation at lower and safer ventilator parameters. Ventilators carry an as yet undefined risk to the patients. If carbon dioxide exchange can be partially accomplished through a benign and safe extracorporeal device, settings on pulmonary ventilators can be limited to within more safe limits.

Methods Employed and Major Findings:

1. The Membrane Lung

There are at this time no problem areas remaining which are traceable to

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quality of silicone rubber membranes. The double layer membrane casting machine developed in this laboratory permits us to cast multi-layer membranes with or without fabric reinforcement. These membranes are exceptionally strong. Similarly, it has been possible to tailor make membranes of extreme thinness for high flux respiratory carbon dioxide elimination from the membrane lung. Thus, the standard membrane may be 50 microns in thickness, a carbon dioxide exchange membrane may be only 25 microns thick.

The following silicone rubber membranes have been cast using pure polysiloxane gum homopolymers.

a. Three layer membrane, with blood contacting layer made of polytrifluropropylsiloxane.

b. Three layer membrane, with blood contacting layer made of polymethylphenylsiloxane.

c. Three layer membrane, with blood contacting layer made of polymethylvinylsiloxane.

d. Three layer membrane with blood contacting layer made of polydimethylsiloxane.

The carbon dioxide exchange rate for all of the above polymers is in the acceptable range. Oxygen blood gas diffusion rate is such that a 2½ square meter membrane lung has sufficient capacity for routine open heart procedures. Somewhat higher surface areas are required for long term extracorporeal pulmonary assistance in acute respiratory failure.

Much has been written in recent biomedical literature about the effect of carbon added in polyurethanes (electrolour), the salutory effects of applying a negative potential to a vascular prosthesis, the nature of improved biocompatibility through addition of certain carbons, and more. The following special membranes were prepared and studied.

e. We have prepared fabric reinforced silicone rubber membranes of four discrete layers and enriched one of the layers with 25% acetylene black carbon. The blood contacting layer was polymethylvinylsiloxane without silica filler or carbon.

e. We have prepared fabric reinforced silicone rubber membranes of four discrete layers and enriched one of the layers with 25% acetylene black carbon. The blood contacting layer was polymethylvinylsiloxane without silica filler or carbon.

f. We prepared a two layer silicone rubber membrane with a blood contacting layer consisting of polymethylvinylsiloxane enriched with 25% acetylene black carbon.

g. We prepared a three layer standard silicone rubber (SSR) membrane where the second layer was polymethylvinylsiloxane enriched with 25% acetylene black carbon and a blood contacting side was of SSR with silicone filler.

A. Clinical Applications

We believe that a great number of patients ultimately requiring blood respiratory gas exchange represent complications of intensive respiratory care.

No patients have been treated by ECMO at the Clinical Center during the past year. Six possible candidates that we have been consulted about were referred to out of state institutions as there is no local clinical group pursuing membrane lung bypass at this instant.

The NCI patient with acute leukemia and acute respiratory failure bypassed at the NIH for 10 days over three years ago remains in excellent health.

B. Animal Research

Membranes produced in this laboratory have undergone testing in animals by the arterio-venous shunt technique. Spiral membrane lungs of 0.6 m^2 were prepared from study membranes and were connected to the arterio-venous shunt. No heparin was given once bypass was begun. We measured changes in the resistance to blood flow, changes in blood platelet count, white blood cell count, and differential count. Lack of any change in the above parameters implied excellent blood compatibility.

a. Polytrifluropropylsiloxane appears to be highly thrombogenic for use in the membrane lung. Interestingly enough, this homopolymer was thought to offer significant throboresistance by the inferior vena cava ring test. Our studies cast serious doubts on possible use of this homopolymer in the membrane lung.

b. The membrane lungs made of polymethylphenylsiloxane showed in all six out of six studies significant rise in perfusion resistance, and fall in blood platelet count. Three membrane lungs clotted.

c. The membrane lungs made of polymethylvinylsiloxane showed a rise in perfusion pressure or clotted in three out of six membrane lungs studied. Interestingly enough, methylvinyl based polysiloxane membranes are at present nearly exclusively used in medical applications primarily because of the more efficient cross linking effected through the use of vinyl side groups.

d. All ten out of ten membrane lungs made of polydimethylsiloxane showed no rise in perfusion pressure and no falls in platelet count during the six hour study period, and remained patent (without heparin) for 24 hours.

e. The four layer membrane made as under "c" but modified with the addition of carbon, showed significantly improved performance. No membrane lungs clotted and the platelet count remained at baseline values for the duration of the study.

f. The membrane lung with a two layer membrane with acetylene black containing polymethylvinylsiloxane in direct contact with blood showed some unusual response to a single bolus of heparin. When 10,000 units of heparin was administered in a single bolus on these three, the blood platelet count rose immediately, and continued to rise to twice the baseline values (1,170,000 per cubic millimeter). This degree of thrombocytosis has not been observed with any other membranes studied to date and have not been reported previously in the literature. This phenomenon is currently being investigated.

We found no change in white blood cell count or differential count in this group of membrane lungs.

g. Membrane lungs prepared of standard silicone rubber with silica filler, modified through addition of carbon have shown absence of change in white blood cell count, and no change in differential count, similar to findings under "f" above.

White blood cell changes have been universally seen in all membrane studies in the past (excepting as noted in this report) both in the thousands of hemodyalises performed daily, and in all membrane lung perfusions. We do not understand the importance of the mechanism of these changes. It is reasonable to assume that changes in white blood cells are undesirable, and that they indirectly reflect "impaired" blood compatibility.

We believe to have at this time a "handle" in the form of carbon, by which white blood cell response after blood exposure to membrane can now be modified.

C. Membrane Lung Priming

No membrane lung comes primed, ready for use at a moments notice. Unfortunately for optimum blood compatibility, a membrane lung has to have all air bubbles removed beforehand.

We have developed a novel vacuum priming system where a membrane lung can be perfectly primed in as short a time as three minutes. The key to this success lies in totally degassing the priming solution of all dissolved gases, and then priming the membrane lung in a vacuum of 2-3 Torr.

The total amount of residual air is less than 0.005 cubic centimeters at STP.

2. The Carbon Dioxide Membrane Lung

A membrane lung as well as the natural lung are expected to provide for both carbon dioxide and oxygen exchange in a proportion depending on the respiratory quotient (RQ).

All current use of the artificial lung assumes priority gas exchange for oxygen with little attention paid to carbon dioxide exchange. This thinking is excusable because oxygen deficiency is rapidly fatal while carbon dioxide exchange could be compromised repeatedly without serious consequences for many minutes and even hours.

Oxygen transport with a membrane lung requires passing virtually the total cardiac output through a membrane lung every minute. The peculiarities of carbon dioxide biochemistry makes it possible to effect total carbon dioxide exchange, however, with a minute blood flow of approximately 20% of cardiac output.

We believe that future clinical care of the patient with acute respiratory failure, and that of any patient sick enough needing the use of a respirator, may be greatly changed by the availability of the high efficiency carbon dioxide membrane lung. Such a device could reduce blood PCO_2 from the level of 40-50 mm Hg. to as low as 20 mm Hg. in a single pass. One liter of blood flow per minute thru the membrane lung can remove the total CO_2 produced by the whole body.

We believe that by exchanging carbon dioxide through the membrane lung we can maintain ventilator settings in a safe range to maintain low peak respiratory pressure by reducing inspiratory tidal volume.

These hypothesis were tested in a preliminary study in unanesthetized, unrestrained sheep, with the membrane lungs placed and the arterio-venous circuit. We were able to show that minute ventilation, and tidal volume, was greatly dependent on carbon dioxide exchange through the membrane lung. When virtually all carbon dioxide was eliminated through the membrane lung, the respiratory rate fell to 0-10 per minute. The minute ventilation fell by 50-100%, and the tidal volume was 0-50% of baseline values.

We believe that these findings can be applied in a select patient population with acute carbon dioxide retention, such as in the case of uncontrollable bronchopleural fistula. We cannot speculate at this time on the future use of this mode of carbon dioxide elimination on chronic lung patients with hypercapnea, cystic fibrosis, etc.

Significance to Biomedical Research and the Program of the Institute:

There is a well defined need for improved artificial lungs in various clinical settings. The artificial lung may be used either alone, or

combined with a blood pump. The former is of greatest use in pulmonary problems, whereas the latter could be suitable in cardiac assist (right and left side heart assist or combined assist).

The need for improved biopolymers is related to present day need to use large doses of heparin. Unfortunately, large doses of heparin also contribute to bleeding problems, hence the desire for improved blood compatible membranes.

Our studies have shown that a silicone polymer presently used in cardiovascular applications is not highly blood compatible, contrary to what has been commonly thought. Polydimethylsiloxane without silica filler was shown by us to be superior to other silicone polymers, and is now the biopolymer of choice for a wide variety of cardiovascular applications.

We have also shown that the addition of acetylene black carbon greatly modifies the behavior of silicone rubber membranes. White blood cells and granulocyte changes no longer are seen with these membranes. Similarly, under certain well defined circumstances these membranes can maintain peripheral blood platelet count (without heparinization) and thereby virtually eliminate hemhorrage from the list of bypass complications.

These new biopolymers can find application not only in devices listed above, but also in hemodialysis, heart valves, vascular grafts, blood catheters, and other blood contacting devices. Its wide use could result in significant reduction in morbidity and mortality.

The use of carbon dioxide membrane lungs without heparin (or only a limited need for heparin) could significantly effect clinical care of patients on mechanical ventilators.

Proposed Course:

1. We will explore other membrane configurations using acetylene black carbon enriched silicone rubber membranes.

2. A program to develop a novel type of membrane lung suitable for long term implantations will be continued. This will include the preparation of new types of surfaces of membranes, and new designs, to make a reliable compact unit.

3. The various aspects related to the carbon dioxide membrane lung will be explored further. This will include optimal configuration to effect high carbon dioxide fluxes and the preparation of special ultrathin silicone rubber membranes.

Publications:

1. Kolobow, T., and Stool, E. W.: Ultra-thin Silicone Rubber and Silica Free Silicone Gum Rubber Membranes for Use in the Membrane Lung. Proceedings, International Conference on Membrane Lung Technology and Prolonged Extracorporeal Perfusion, 1976, In Press.

2. Kolobow, T., Stool, E. W., and Pierce, J.: Long Term Perfusion with the Membrane Lung in Lambs. Proceedings, International Conference on Membrane Lung Technology and Prolonged Extracorporeal Perfusion, 1976, In Press.

3. Kolobow, T., Tomlinson, T., Gattinoni, L., and Pierce, J.: Platelet Response to Long Term Spiral Coiled Membrane Lung Bypass without Heparin Using a Carbon Silicone Rubber, Membrane. Am. Soc. Artif.Intern. Organs, 1976, In Press.

4. Newball, H. H., Stool, E. W., and Kolobow, T.: Follow-up Respiratory Function of a Patient Treated with a Membrane Lung. Amer. Rev. Resp. Disease, 112: p, 725-731, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01405-03 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Analysis of Microcirculation by Coherent Light Scattering

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI : M. D. Stern G:DIR

OTHERS: R. L. Bowman Chief, Lab. of Technical Development LTD NHLI

COOPERATING UNITS (if any)

Laboratory of Experimental Therapeutics, NHLI; Surgical Neurology Branch, NINCDS; Biomedical Engineering Program, NIGMS; Renal Division, Southwest Texas Health Sciences Center, San Antonio; and Electrical & Electronic Eng. Section, BEIB.

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is the development of a non-invasive method of measuring tissue blood flow by means of the doppler scattering of laser light. A prototype instrument has been developed which is capable of monitoring blood flow in skin and the surface of internal organs. Preliminary experiments have shown excellent results in monitoring the effects of pharmacologic agents on the blood flow of rat renal cortex, measured continuously in time, in mapping areas of infarction on the cerebral cortex of the rhesus monkey, and measuring blood flow in human skin. Work is in progress to calibrate the method against radioactive microspheres in the renal cortex, and measure flow in the renal papilla.

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PHS-6040
(12-75)

Project Description:

Objectives: To continue development of techniques and applications of a method of continuously measuring the blood flow through small regions of tissue by analysis of the spectrum of coherent light which is doppler-scattered from tissue. The prototype method appears to be capable of studying surface blood flow of a great variety of organs, with potential research applications in vascular physiology and pharmacology, and clinical applications in the study of peripheral vascular disease, burns and grafts, monitoring of tissue perfusion at surgery and under influence of anesthesia etc. Specific objectives at this stage are the improvement of the prototype apparatus and the demonstration of applications to a variety of experimental problems. Commercial development of this instrument is anticipated, making possible more extensive investigation of new applications.

Methods:

(a) Instrument Development

The prototype apparatus for monitoring tissue blood flow is continuing to be improved. We have found that an appropriate index of perfusion can be obtained by calculating a weighted root-mean-square bandwidth of the doppler spectrum of scattered light. Originally this was done by laborious computation from whole spectra. We have developed an analog processor which computes this flow index continuously as a function of time, without the need for a spectrum analyzer. We are currently developing a digital version of part of this electronics, to improve precision and reliability. We are collaborating with an instrument manufacturer on the development of an engineered prototype of the optical system, which would be portable and capable of delivering the measuring beam at a variety of angles and locations, and of being used in an operating room and sterile field.

(b) Studies of Brain

We are collaborating with the Surgical Neurology Branch, NINCDS, to use the laser doppler system to study perfusion of the cerebral cortex of the rhesus monkey, using a preparation chronically equipt with a transparent plexiglass calvarium.

(c) Studies of Kidney

We are undertaking a collaboration with the Laboratory of Dr. Jay Stein, San Antonio, Texas, to use our instrument in studies of renal physiology. This includes comparing our method with the radioactive microsphere method, and use of the laser doppler method to study blood flow in the papilla of the kidney, where there is no practical method at present.

Major Findings:

1. The root-mean-square flow parameter appears to be quite successful as an index of flow in several tissues, under a variety of interventions. We have constructed an analog system which computes this parameter continuously. The analog system is quite convenient. However, it suffers from some problems of linearity near zero flow, because of the difficulty of taking square roots with analog circuitry. For this reason we are developing digital circuitry for part of the computation.

2. Good flow signals have been obtained from the surface of the cerebral cortex in the monkey, using the preparation described above. Because of the local variations in vascular geometry from point to point on the brain (as opposed to kidney for example), it is more difficult to get quantitative information from the flow parameter. However, we have been able to map the region of cortical infarction produced by ligation of the middle cerebral artery, even in cases where the occlusion is only partial. There seems to be a possibility that the superficial pial vessel behave differently in response to CO_2 than bulk brain tissue vessels. We have not determined whether this is really the case, or represents an artifact caused by the limited bandwidth of our present instrument.

3. An arrangement has been made with an instrument manufacturer to produce several prototype instruments for use in various applications proposed by other investigators throughout the country. These include studies of the dynamics of pulmonary capillary flow, studies of blood flow in bacteremia. study of flow in the renal papilla and clinical applications to the surgical patient. Other experiments in preparation include study of the perfusion of the myocardium. This may be difficult because of motion artifacts in contracting cardiac tissue.

4. A clinical protocol exists for studies within the clincical center of NIH. So far these have not been done because of limiting availability of the single instrument which has been dedicated to animal studies.

Significance of the Program to the Institute:

Laser doppler spectroscopy appears to be a promising method of studying microcirculation of tissues. It has potential applications not only in the laboratory, but in the clinical study of peripheral vascular disease, the screening of vasoactive drugs, and the monitoring of patients with unstable circulatory systems.

Proposed Course:

1. Continue development of the instrument, in collaboration with BEIB and industry.

2. Continue study of cerebral blood flow in collaboration with NINCDS.

3. Calibration against radioactive microspheres in the renal cortex of the rat or dog.

4. Study of blood flow in the renal papilla of the Munich Wistar rat.

5. Collaboration with other investigators interested in developing new applications for the instrument.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01406-12 LTD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Fluorescent Complexes of Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. F. Chen Medical Officer LTD NHLI
OTHER: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/3	1/3	

SUMMARY OF WORK (200 words or less - underline keywords)

This project concerns the interaction of proteins with fluorescent ligands used as probes to study macromolecular structure. Present work includes: 1. Binding studies on the interaction of cationic fluorescent dyes with alcohol dehydrogenases and serum albumins. Such dyes appear to bind in areas distinct from those occupied by more commonly used anionic fluorescent probes. 2. Spectral characterization of quinacrine mustard conjugates of proteins in order to establish the usefulness of this dye in protein chemistry as well as nucleic acid and chromosome studies. 3. Preparation and characterization of protein conjugates containing a new dye, N-105, made by Walter Stewart of NIAMDD. The dye is designed for fluorescence microscopic localization of nerve processes following microinjection into the axoplasm of individual nerves.

PHS-6040

Objectives: The aim of this project is to study and characterize the fluorescence of complexes of proteins containing dyes. Some of these dyes are useful as fluorescent probes of protein structure, and others may be useful for protein assay or other purposes. Since dye-labeling of macromolecules is a significant part of fluorescence spectroscopy, advances in this area enchance the usefulness of fluorescence methods in general.

Methods Employed: The characterization of fluorescence of dye protein complexes was achieved through the use of spectrofluorometers, lifetime apparatuses and polarization instrumentation already present in the laboratory. Most of the proteins used in the study were all available commercially in crystalline form except for serum albumins of some species, which were available as Cohn Fraction V preparations.

Major Findings: The work with quinacrine mustard-labeled protein conjugates was completed. Quinacrine mustard (QM) is a derivative of quinacrine (atebrine) which reacts with primary amine groups in macromolecules to give a fluorescent derivative. Although widely used in studies on DNA and chromosomes, little work on protein-labeling by QM has been performed. We found that many proteins were readily labeled by QM, and the conjugates could be separated by Sephadex filtration. Quantum yields ranged from .1 to .3, and lifetimes ranged from 4 to 13 nsec. Lifetimes were longer, and quantum yields were lower, for DNA or RNA derivatives of QM, suggesting that in nucleic acids some QM molecules were markedly quenched. The spectra and polarization of the conjugates were measured, and it was shown that the fluorescence was affected by pH or denaturants in such a way that QM could be considered a fluorescent probe of structure. In a paper which was published, it was pointed out that QM has the following unusual features: 1 - An absorption spectrum matching the major mercury lines at 436, 366, and 254 nm. 2 - A positive charge; most fluorescent labels are anionic or neutral. 3 - Water solubility.

We continued work on the new dye synthesized by Walter Stewart, NIAMDD, called N-105. The dye was designed for use for microinjection into nerve axons in order to trace nerve processes by fluorescence microscopy. The dye is N-(m-vinylsulfone phenyl)-4-aminonaphthalimide-3,6- disulfonic acid, and reacts well with proteins, presumably at sulfhydryl and amino groups. Characterization of the fluorescence (spectra, polarization, lifetimes, quantum yields) of various conjugates of N-105 was carried out, and a draft of a paper was prepared. However, Dr. Stewart wishes to delay publication until the more general communication of the synthesis of the compound and related compounds which he has developed appears.

The properties of Auramine O, bis(p-dimethylaminophenyl)-methylenimine hydrochloride, were studied because this is one of the few cationic fluorescent probes which have been successfully used for proteins. Although it has been reported in the literature that Auramine O is nonfluorescent in water and other solvents and becomes fluorescent only when bound to liver alcohol dehydrogenase, we were able to detect fluorescence from the

dye dissolved in a number of solvents, and to show that the dye binds to yeast alcohol dehydrogenase and serum albumins, becoming fluorescent in the complexes. From equilibrium dialysis and fluorescence titrations, we showed that the yeast enzyme binds 2 moles of dye per mole of protein, with a low association constant of $1.2 \times 10^4\ M^{-1}$. For 7 serum albumins, only 1 site was found and the association constant ranged from 0.23 to $1.9 \times 10^4\ M^{-1}$. Spectra and quantum yields were measured. It was concluded that the LADH-Auramine O complex was unique mainly in its higher association constant. A review of the literature on fluorescent probes shows that enzymes often bind anionic fluorescent probes such as ANS, but infrequently bind cationic probes in their active sites. The only exceptions are enzymes which are adapted to cationic substrates: examples are acetylcholinesterase, and trypsin. Because liver alcohol dehydrogenase binds a variety of cationic probes strongly, this suggests that the enzyme's natural substrate may be some cationic compound, rather than ethanol.

A paper has been prepared on these studies and submitted for publication. An abstract has been accepted for a talk to be given at the meeting of the American Society of Biological Chemists in June.

Significance to Biomedical Research and the Program of the Institute:

The use of dyes as fluorescent probes of protein structure is a large reason why fluorescence spectroscopy has become so useful in biochemistry. Previous work in this laboratory had contributed to the usefulness of the fluorescent probe method. The present work continues that tradition. The work on cationic probes of proteins may well open up a new area of investigation, since practically all previous work on protein probes utilized anionic probes.

Proposed Course:

We intend to pursue the use of cationic probes in protein studies to see whether anionic and cationic probes bind at different sites. Present evidence suggests that a given protein may have separate sites for anionic and cationic ligands. From energy transfer studies, it should be possible to determine the distance between such sites. Other cationic dyes such as proflavin, and acridine orange have been found to bind to the alcohol dehydrogenases; further work in this area will define the size of the binding site and its distance from the coenzyme site. Compounds such as quinacrine mustard might show affinity labeling of cationic sites, which would then be unavailable to bind dyes such as Auramine O. Such studies will enhance our understanding of the use of cationic dyes as well as our knowledge of protein structure.

Publication: Chen, R. F.: Fluorescence of Quinacrine Mustard Conjugated to Proteins, Arch. Biochem. Biophys. 172, 39-50, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01407-13 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Applications of Fluorescence in Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. F. Chen	Medical Officer	LTD NHLI
OTHER:	M. Gottlieb	Senior Investigator	LPB NIAMD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/3	1/3	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to demonstrate novel applications of fluorescence spectroscopy in problems of biochemical interest. Recent work has included: 1. The study of the binding of the fluorescent antibiotic pimaricin to red cell membranes by stopped-flow fluorescence. Since pimaricin fluorescence enhancement is thought to occur upon binding to membrane cholesterol, the biphasic kinetic plots suggest the presence of two types of cholesterol pools. 2. The rate of labeling of proteins by fluorescent dyes was found to be measurable by stopped-flow fluorescence. Conjugates made by reaction with fluorescamine and quinacrine mustard have higher fluorescence yield than the free dyes. The rates of reaction are altered by competing ligands and modifications of protein structure.

PHS-6040
(12-75)

Objectives: The purpose of the project is to demonstrate the usefulness of new methods or techniques of fluorescence spectroscopy by applying them to problems of interest in biochemistry.

Methods Employed: In the work described below we have specifically used the stopped-flow fluorescence apparatus which was described by us several years ago. The kinetic studies were supported by measurement of fluoresence parameters such as spectra, quantum yields and lifetimes, using the methods we previously described.

Major Findings:

1. The collaboration with Dr. M. Gottlieb, a study was done on the interaction of the fluorescent antibiotic, pimaricin, with red cell membranes (ghosts). The quantum yield is greatly enhanced upon binding, and it is thought that the binding occurs in areas where cholesterol is located. The large enhancement of fluorescence was followed after rapidly mixing the pimaricin with red cell membranes. The time course of the fluorescence enhancement clearly showed two components. Half-times of the order of 50 msec and 1 sec were found. The results suggest that there were two cholesterol pools which differed in their accessibility to pimaricin. Dr. Gottlieb prepared "inside-out" membranes, using an enzymatic method, and we found that the kinetics were markedly altered. A variety of control studies were performed using lipid preparations instead of the membranes, and using various concentrations of the reactants. These studies are germane to the objectives of the project, since not only is stopped-flow fluorescence a relatively little used technique, but also the use of membranes in a stopped-flow experiment is uncommon. The results of this study will be incorporated into a manuscript presently in preparation by Dr. Gottlieb.

2. Stopped-flow fluorescence methods were also used to study the rate of labeling of proteins by fluorescamine, a reagent which is not fluorescent until it reacts with free amino groups. The purpose of the experiment is to show that such kinetic studies are useful in structural studies of proteins. It stands to reason that reactive groups on the protein can be made either more or less accessible by conformational changes or by binding of ligands such as coenzymes. Such changes in accessibility will be reflected in the reaction rates with fluorescamine. Results to date show that the reaction rates are readily measured, since the half times fall in the range of 0.1 to 1 sec. A problem with the method is such as dioxane or acetone, and mixing such a solution with a protein may in itself cause structural change. However, we have found that fluorescamine is fairly stable in mixtures of acetone and water in the absence of base, so that the amount of organic solvent needed is much reduced. A few experiments have been performed with quinacrine mustard instead of fluores camine, since the former dye is water soluble. However quinacrine mustard

is fluorescent, so that the rate of reaction with proteins can be followed only when the quantum yields of the free and bound dye are significantly different.

Our results show that the rates of reaction with fluorescamine are very different for different proteins, and the time course of the fluorescence increase is usally complex, since the reacting groups on the proteins are inhomogeneous.

Significance to Biomedical Research and the Program of the Institute:

These studies have produced results of interest to membrane biochemists and those interested in protein structure. The usefulness of stopped-flow methods in fluorescence is illustrated; many of these methods were developed in this laboratory.

Proposed course:

There are several problems which should be amenable to study with methods and technqiues pioneered in this laboratory.

1. We plan to complete the work on stopped-flow fluorescence of fluorescamine-protein labeling.

2. The use of aqueous snows in phosphorescence studies now enables us to study proteins denatured in guanidine or urea. We have already obtained preliminary data on the phosphorescence quantum yields of proteins which are fully denatured and will complete this work. The results are relevant to the question, "how much influence does the three-dimensional structure of a protein have on the phosphorescence yield and spectra, as opposed to neighboring group influences?"

3. Further studies on the influence of metal ions on protein fluorescence are planned. In the first type of study, the influence of protein binding on the fluorescence of ions such as thallous, europium, and terbium will be measured. In another type of study, the use of metal ions such as Cs^+ as fluorescence quenching probes of accessibility will be investigated.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL Q1408-11 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Methodology in Fluorescence Measurements

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. F. Chen Medical Officer LTD NHLI

Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/3	1/3	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to refine and develop methods used in fluorescence spectroscopy as applied to biomedical research. At present, work is focused in these areas: 1. Measurement of fluorescence lifetimes using standards single photon counting, and computer evaluation of goodness of fit. 2. Orientation of biomolecules by flow and other means, for polarization studies. 3. Evaluation of the reaction kinetics of fluorescamine, a common fluorogenic reagent. These reaction rates are studied by stopped-flow methods, and are relevant for the use of fluorescamine in automated procedures.

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PHS-6040
(12-75)

Objectives: The long range purpose of this project is to contribute to the refinement and development of methods used in fluorescence spectroscopy, as applied to biomedical research.

Methods Employed: In developing apparatus for specific purposes, we usually start with a commercially available instrument and modify it, using shop facilities, to enable it to be used for special measurements. In evaluating methods or reagents, existing instrumentation previously devleoped in the laboratory are applied for the measurement of reaction rates, fluorescence yields, lifetimes, and polarization.

Major Findings:

1. In the measurement of fluorescence liftetimes with the commercially-available ORTEC 9200 nanosecond spectrometer, a common procedure is to compare the experimental decay curve with artifically generated curves representing different lifetimes. We find it more convenient and accurate to use fluorescence lifetime standards, developed previously in this laboratory, and recent work focused on computer estimation of the goodness of fit for comparing the experimental and standard decay curves. This has involved developing computer programs which quantitate the differences between the curves. This has been done using the PDP-10, and MLAB, a program for modeling and graphics.

2. We have been studying the rate of reaction of fluorescamine with various amino acids and other amines, using the technique of stopped-flow fluorescence. It is generally assumed that fluorescamine reacts within milliseconds with primary amines to give a fluorescent product. The reagent is therefore used in automated, rapid, fluorometric assays. Our studies of reaction rates of fluorescamine with various amino acids have shown that the reaction rates vary with concentration of the reactions, as expected for second order reactions, and the rate constants are quite slow for the acidic amino acids, glutamic and aspartic acids. Depending on the concentrations employed, the time required for 90% completion of reaction may be as long as 2 minutes. Also, fluorescamine has been said to be unstable in water, but we find the reagent unstable only in the prescence of base. The rates of reaction of fluorescamine with a number of amino acids have been obtained. These data can be used to determine the optimum conditions for fluorescence development in fluorescamine procedures.

3. The possibility of flow orientation of macromolecules by means of a modification of commercial spectrofluorometer was investigated. A review of the literature on flow dichroism and flow birefringence was undertaken. Some experiments were carried out to see if orientation could be observed by fluorescence polarization of dyes attached to DNA which was flowed through a 1 mm capillary. The literature suggests that shear rates of 3000 sec^{-1}

are needed to obtain useful orientation of DNA. Slight change in the polarization ratio was observed with this crude preliminary instrumentation, and we are encouraged to try other methods. Some orientation seemed to result when DNA-dye solutions were rapidly swirled with a magnetic stirrer operated by a motor and magnet installed beneath the sample chamber.

Flow oriented fluorescence polarization can supply several types of information not readily obtained by other methods. For instance, the "intercalation" concept wherein mutagenic dyes insert between base pairs of DNA was established through flow oriented fluorescence polarization methods. Also, the relative orientation of dyes on an elongated macromolecule could be determined, as well as the relative orientation of two different dyes on a protein or nucleic acid. Oriented smaller molecules could give information on the direction of the transition moments for absorption and fluorescence.

Significance to Biomedical Research and the Program of the Institute:

The first spectrofluorometer was developed in this institute some 20 years ago, and fluorescence spectroscopy became available to many laboratories largely as a result of that development. By continuing to advance methodology in fluorescence spectroscopy, we continue to refine one of the most useful tools in biomedical science.

Proposed Course:

Although the ORTEC nanosecond spectrometer is now being used in a number of laboratories, this instrument is not easy to use. We wish to continue to explore ways to simplify the measurement of lifetimes and nanosecond polarization. In conjunction with engineers from the computer center we will try to get digital data sent directly onto discs through the phone lines rather than use the present system, which is to first make paper or magnetic tapes, which are then sent over the lines.

The fluorescamine study is nearly complete, with regard to obtaining data on reaction rates. It is clear that the detailed mechanism of the fluorescamine reaction is not fully understood. For instance, it is not known why the fluorescence quantum yields are so different for the different amino acid derivatives of fluorescamine, since the postulated fluorophor is the same in each case. Further examination of the quantum yields, absorption and fluorescence spectra, and the use of various model primary amines may provide more insight into the reaction.

The problem of providing a flow orientation system which can fit into the spectrofluorometer will be approached by 1 - Investigating the utility of stretched polyvinyl alcohol films. 2- Testing the suitability of commercially produced flow dichroism apparatus. 3- Possibly testing the utility of arrays of capillaries.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01409-05 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Develop Methods for Rapidly Assaying Bacterial and Mammalian Cell Growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. Carmeci Electronic Engineer LTD NHLI

COOPERATING UNITS (if any)
Medicine Branch, National Cancer Institute, N.I.H.
Hematology Service, Clinical Center, N.I.H.

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

The work involves two areas of endeavor, the first is to develop methods for rapidly measuring various paramters of bacterial and mammalian cell growth and the second is to demonstrate the instrumentation methods and techniques and facilitate their use in areas of endeavor not heretofore utilized. Using the capillary tube technique and spatial frequency analysis methods the growth of bacteria, mycoplasma and bone marrow stem cell has been investigated. Capillary flow has also been applied to the study of red blood cell fragility.

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Objectives:

The objectives of this project were 1) to find a method for assaying the growth of bone marrow stem cells cultured in a test tube and 2) utilize capillary flow techniques for measuring red blood cell fragility. The details of these investigations follow.

A. Method for assaying bone marrow stem cells.

A new method of culturing human bone marrow stem cells has been developed. The cultures are grown in large plastic test tubes and the stem cells grow more profusely and reliably than in Petri dishes or capillary tubes. This was shown in a series of tests with human stem cells wherein samples were extracted into capillaries at various intervals during their growth in large tubes.This method caused us to investigate the possibility of detection assay within the larger test tube directly. The colonies are very few in relation to the total volume of the culture flask, therefore, to detect these by light transmission would be very difficult. Although the media scatters a lot of light, light scattering seemed to be the easiest method ofr detection and assay.

When the colonies are small, grown in a three dimensional solid media, and appear against a bright background of light scattered from colloidal media conventional colony counters fail. Computerized image analysis is elaborate and expensive. A simple approach has been developed for quantitating colony growth by using spatial frequency analysis to detect small colonies against a uniform bright background. Spatial frequencies are converted into temporal frequencies by chopping the image with a rotating optical pattern wheel, a method developed for use in missile guidance systems to detect small emitting targets against bright clouds. A prototype system, has been developed and applied to monitoring the growth of granulocyge colonies in methylcellulose medium with bone marrow suspensions, with good results. A contract for a developmental model designed on the basis of a prototype instrument made in this laboratory was let to the Naval Weapons Center, China Lake, California. The first of three models has been delivered and is presently undergoing evaluation in the N.C.I.

B. Measurement of red blood cell fragility.

A simple and accurate method for the determination of cell osmotic fragility has been investigated. The method uses the Poiseuille's parabolic flow pattern together with gravity to retain cells in a long capillary tube while a solution with decreasing osmolarity is passed through the tube for hemolysis. Hemoglobin released from the cells is quickly removed by the axial flow pattern and monitored with a spectrophotometer for recording the hemolysis curve. Comparisons have been made with present methods and

the advantages are: 1) Sample size is small (2 ul), 2) Output is in a non-integrated form, 3) No sample preparation is necessary, 4) Accuracy and reproducibility is higher and 5) The method is amenable to changing parameters.

Significance to Biomedical Research and The Program of the Insitute:

The assay of bone marrow stem cells can be applied to other types of mammalian or bacterial colonies that can be grown in a test tube environment. The measurement of red blood cell fragility offers the above mentioned advantages and this method can facilitate osmotic fragility studies in other types of cells.

Proposed Course:

A. This project is complete.

B. Completion of the study and publication of the method.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01410-02 LTD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Blood Gas Monitoring for Extended Periods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. G. Vurek	Senior Investigator	LTD NHLI
OTHER:	T. Kolobow	Chief, Section on Pulmonary and Cardiac Assist Devices	LTD NHLI
	T. Clen	Electrical Engineer	BEIB DRS

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves the development of a system to measure PO_2 and PCO_2 in extracorporeal circuits for more than 24 hour periods without blood loss. Picomole amounts of O_2 and CO_2 are extracted from the circulation with a special probe. Oxygen is analyzed by amperometry and CO_2 is measured by calorimetry of the reaction of CO_2 with LiOH. Tests have shown the system to be stable within 5% over 25 hour periods.

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PHS-6040
(12-75)

Objectives: The objective of this project is to make a system which will provide continuous information about blood PO_2 and PCO_2 during extracorporeal circulation procedures. The system does not require removal of blood from the circuit and can operate without calibration more frequently than once per day.

Methods Employed: There are three parts to the system: the sampling probe, the analytical instrument, and the data handling circuits In order to eliminate blood sampling, a small amount of oxygen and carbon dioxide are removed, in proportion to their blood partial pressures, from the extracorporeal circuit by the sampling probe. The gases are transported by a flow of nitrogen carrier gas from the probe to the analytical instrument. Oxygen is measured amperometrically because this technique is sensitive (10^{-12} mol sec^{-1} yields 2.6 μA) and quantitative. Carbon dioxide is measured by measuring the heat of reaction of CO_2 with LiOH, a technique which yeilds a sensitivity of $1x10^{-12}$ moles. The partial pressure of blood gases depends on blood temperature; their diffusion through the sampling membrane is also temperature dependent. A thermistor in the sampling probe provides information about the sample temperature to correct the transducer outputs to standard temperature (37°C). The data processing unit provides baseline, temperature, and other correction factors to the transducer, and displays and records the data.

Major Findings:

The principal effort of the current years work has been to improve the probe design and improve the performance of the analytical system. The function of the probe is to extract O_2 and CO_2 from the extracorporeal circuit proportional to the partial pressures without being influenced by changes in blood flow velocity and being constructed of blood compatible materials with good hydrodynamic design. We have explored several materials which could give adequate gas flux without excessively depleting the boundary layer of blood adjacent to the probe. The improvements to the analytical system have been centered around trying to achieve a better understanding of the conditions required to obtain stable sensitivity from the CO_2 apparatus and to improving the plumbing system's reliability and convenience.

The probe must extract about 10^{-12} mole/sec. of O_2 and perhaps five times that amount of CO_2 in order to provide enough gas to transducers for precise measurement. Silicone rubber has excellent biocompatibility but is too permeable so that flow effects would be severe. Earlier work suggested that a membrane of vapor deposited material, Parylene C, would have the necessary low permeability, and thinness needed for reasonable response time. However, the coating was too fragile when applied to the flexible silicone rubber substrate. We decided to mold a segment of silicone rubber tubing with a thin (60-100 μm) wall in one region against which an external probe could be placed The probe was designed so that

membranes with good gas transfer properties could be tested without imposing a biocompatibility requirement. We tested membranes made of polyethylene, Mylar and Polyethersulfone. The latter is very good because it has a relatively low coefficient of change in permeability with temperature, being 2% K^{-1} in contrast to 4.7% K^{-1} observed with polyethylene. Its principle disadvantage is its tendency to tear at the desirable thickness, 8 μm. Mylar, even at 2.5 μm thickness is too impermeable. With the polyethylene membrane, and a 1 cm diameter tube, the sensitivity to flow was such that at least 95% of the maximum response was obtained for flows greater than 3.8 cm/sec. The rise time (10%-90%) response to a step change in PO_2 was 59 seconds.

The various tubing connections were changed to make them tighter and where necessary, easily disconnected. Swagelok fittings were put aside and either soldered joints or a special O-ring connectors were used. In addition, the air-motor driven valve was replaced with a diaphragm type valve that requires much less electrical power as well as having a small dead volume. We investigated the possibility of using Kel-F tubing in place of the stiff stainless steel tubing but found it to have a tendency to bleed dissolved gases for several hours after installation.

One test of the entire system was made using a sheep and a membrane lung to control the gas pressures reaching the probe. By changing the gas composition in the oxygenator, we could run the PO_2 from about 30 up to 150T and PCO_2 from 18 to 65T. A clot formed on the inside of the probe due to improper heparinization. However, the sensitivity to CO_2 stayed constant during the 25 hour test. Oxygen response was considerably more erratic and this was attributed to air leaks around the probe. *In vitro* tests have shown the system to hold its sensitivity within ± 5% of the mean value over 24 hour periods.

Significance to Biomedical Research and the Program of the Institute:

The addition of PO_2 and PCO_2 monitoring to the list of non-sampling methods improves the monitoring capabilities for extracorporeal circulation procedures. If this system is incorporated in the venous line to the oxygenator, an estimate of blood pH can be made if the oximeter data is included.

Proposed Course: Additional investigation is required to understand the nature of the CO_2 - LiOH reaction to make it consistently useful for this technique. In addition, signal handling system will be completed to provide the proper temperature corrections, accommodate the calibration factors, and display the information.

Publications:

Vurek, G. G., and Kolobow, T.: Continuous blood-gas measurement during prolonged extracorporeal circulation. In Oxygen Measurements in Biology and Medicine. J. P. Payne and D. W. Hill, eds., Butterworth, London, 1975, pp 285-296.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01411-10 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Blood Flow Measurement Using Nuclear Magnetic Resonance Techniques

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. Kudravcev	Electronic Technician	LTD NHLI
	R. L. Bowman	Chief, LTD	LTD NHLI
OTHERS:	A. Sances	Professor and Chairman Biomedical Engineering	Medical College of Wisconsin
	J. H. Battocletti	Research Associate	Medical College of Wisconsin

COOPERATING UNITS (if any)
Medical College of Wisconsin, Milwaukee, Wisconsin

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI , NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

SUMMARY OF WORK (200 words or less - underline keywords)

Blood flow will be magnetically polarized, regionally depolarized and detected downstream to measure changes in perfusion of the cerebrum and fingers in animals and humans to evaluate the relative sensitivity of the system at high and low magnetic fields. Non-invasive blood flow measurement by nuclear magnetic resonance techniques will be used.

PHS-6040
(12-75)

Project Description:

To continue to develope the practical aspect of nuclear magnetic labelling technique for biological blood flow measurement, especially cerebral and digital.

Progress: The work is divided between this laboratory and a group under contract to this laboratory at the Medical College of Wisconsin. This group conducts the biological observations using the NMR labelling technique.

In this laboratory, the weak detector field (20 gss) NMR flowmeter, described in last year's report was improved and used to compare its performance with a medium strength magnetic field NMR flowmeter (600 gss). This operation on different field strength levels was performed in order to find the optimal NMR technique for blood flow measurement in vivo, especially for digital blood flow measurement.

A satisfactory level of nuclear induction was obtained in a field 20 gss from simulated blood vessels which were comparable with the size of large vessels in the arm and in the head. The comparative study demonstrates a number of advantages in the use of weak field detector technique. The advantages are as follows:

1. Lack of interference from stationary protons in tissues.

2. Reduction of RF interference which is very strong on high NMR frequencies.

3. Good stability of NMR response.

4. Absence of strong magnetic field drift due to less heat production in the low field magnetic structure.

5. Better signal-to-noise ratio at low flow in comparison to the same measurement using a strong field of NMR detection.

In order to get a sufficient level of blood polarization in the palm of the human hand, for finger blood flow a magnet was modified so that the palm could rest between the poles of the magnet. A high magnetic permeability shield with a 1¼" diameter window to fit the finger was used. An appropriate magnetic field deflection coil and field shimming coil were calculated to eliminate strong field leakage through the window between the polarizing and the weak detector field. The fields should be in close proximity to reduce the loss of blood magnetization by relaxation.

A cross-field arrangement was used to minimize interaction between the polarizing and detection fields. For the enhancement of sensitivity to a

level of satisfactory induction from samll blood vessels and capilaries these new NMR receiving and excitation circuits are used utilizing some global communication concepts. The preliminary result obtained with the new electronic circuit demonstrates its capability to produce satisfactory NMR induction levels from very small blood vessels.

This new model of the finger blood flow meter is in the last stage of construction.

A number of methods used to improve NMR tagging techniques were found during 1975. This electronically improved technique eliminated beat interferences with the detector search RF coil and produced more efficient and cleaner tagging. To improve the performance of the NMR exciter and receiver special RF filters were developed which produced about 10 dB better signal-to-noise ratio at the reciever output.

Significance to Biomedical Research and the Program of the Institute:

Non-intrusive NMR methods can be used to measure peripheral blood flow or blood flow from various organs.

Proposed Course:

One of last year's studies demonstrated the positive possibilities of NMR induction excitation on a chosen level inside the blood vessel by means of precise metering of NMR exciter RF output level. The selective excitation of the NMR induction on chosen levels has the potential for the development of a useful NMR technique to produce meansurment of capillary blood for or blood flow in the blood vessels by choice independently from one another.

Cooperative research with the Medical College of Wisconsin to improve NMR blood flow technique and to produce a more efficient NMR blood flow meter will continue. In our laboratory investigation of double resonance (ERP + NMR) and dynamic polarization to improve the sensitivity of blood flow meters, construction of a practical model of NMR flow meter for peripheral blood flow will be continued.

Publications:

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01412-04 LTD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Discrete Cell Heat Production Measurement as an Indicator of Metabolic Activity.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. L. Bowman Chief, Lab. Techn. Dev. LTD NHLI

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, N.I.H.

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

SUMMARY OF WORK (200 words or less - underline keywords)

The heat evolved from individual cells in a microscopic field modifies the thickness of a condensing film of oil on a thin supporting membrane to allow estimation of heat evolution from individual cells. Granulocyte ingestion of bacteria correlated with increased metabolic heat. Improvement in instrumentation to permit observation by transmitted light and application to specific cell measurements is projected.

Measurement of cell heat evolved on application of transforming agents and metabolic stimulants will be evaluated as an index of specific activity.

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Project Description:

Objectives: To demonstrate the utility of a new microscopic heat detection system to discriminate and quantify the metabolic response of individual cells to metabolic stimulants.

Methods: We are using the system whereby the cells observed under a microscope are resting on an ultra thin membrane exposed to changing vapor pressures of dimethyl silicone monomer that condense and evaporate on the under surface of the cell supporting membrane. The condensation thickness of the silicone film is modified by local heat from the cells and produces interference fringes in the microscope field when observed by reflected light.

Major Findings: The system developed to date requires observation of the cells by incident (reflected) light because the interference fringes are best observed by reflected light. Incident Nomarski interference observation provides good cell images with the limitations and expense of the Nomarski system. This is considered to be less desirable than transmitted light observation so the system has been modified to permit both incident and transmitted light observation to be used.

In the original device the vapor pressure of the interference film forming liquid was modulated by a thermoelectric heat pump placed directly below the specimen film and thus blocked the use of the microscope condensor. The new system has a thinner chamber for the vapor with a thin cover glass bottom which permits illumination by conventional or phase optics. The vapor forming liquid is held in a uniform sheet by being imbibed in a silicone rubber polymer that is transparent. Vapor pressure changes are produced and sequenced by the same heat pump system but the heating and cooling is transmitted by flowing a heat transfer gas thru a heat exchanger on the heat pump and venting the gas on the under surface of the transparent chamber in the space between the chamber and the top of the microscope condensor. Using this system cells can be observed under high power objectives.

The system seems to be more sensitive and uniform than the previous arrangement but application to cell measurements will not be completed until additional part time assistance is available this summer.

The present apparatus has been reconstructed using a standard fluorescence microscope to produce incident illumination with the standard substage illumination system used for transmitted light. Alternate incident and transmitted light or combinations have been used to superimpose the cell image and the heat indication. The old system enclosed the entire microscope in an incubated chamber reducing accessibility. The new system has a built in heater in the slide that holds the chamber and a local draft shield that so far seems adequate.

The use of cholesteric liquid crystals in more purified form to get better resolution of heated regions has not performed to expectations and suggests that present theory cannot be used to predict performance of the system. The unavailability of suitable materials and lack of theoretical basis on which to proceed plus the success of the interference methods makes further investigation of the liquid crystal films unattractive at this time.

Significance to Biomedical Research and the Program of the Institute: Single cell measurements may be particularly important when specific reactor cells cannot be separated from bulk cells. A few reacting cells in a larger batch may be particularly important in oncogenesis, aging, and immuno-reactions.

Proposed Course: Refinement of sample handling, determination of sensitivity, improvement of microscopic image and documentation of results of specific assays.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01413-14 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Instrumentation for the Study of Pre-Steady State Enzyme Kinetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Berger	Research Physicist	LTD NHLI
OTHERS:	P. Smith		LTD NHLI
	M. Marini		Northwestern Med. Sch.
	J. Everse		U. of Cal., San Diego

COOPERATING UNITS (if any)
Ingold Electrodes, Zurich
Thermometrics, Inc., Dover, New Jersey
Biomedical Engineering and Instrumentation Branch, N.I.H.

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2½	2	½

SUMMARY OF WORK (200 words or less - underline keywords)

The development of the 3 millisecond response time thermistor has been completed and incorporated into a stopped-flow apparatus. It will be used in the study of the reaction of carbon dioxide and phosphates with hemoglobin at various stages of oxygenation. Lycra coatings for pH electrodes have proved very effective in protecting electrodes from the effects of protein. This new electrode will be introduced into both stop flow titration and PCO_2 electrode systems. Difficult problems associated with the manufacturer of observation tubes for the high speed optical flow apparatus have been overcome and clean signals with a consistent time resolution of 200 microseconds are presently being obtained. The kinetics of reaction of calcium and EGTA at 25°C and 4°C over the pH range of 6.5 - 7.5 will be continued on this instrument. The laser flash photolysis apparatus for studying the very fast reactions of oxygen in both hemoglobin solutions and red cell solutions has been initiated and the laser has been set up and operated. The optical system and cell hemoglobin studies has been constructed and installed. A system for observing these reactions directly in the red cells is presently under construction. It is based on the "Optisat" principle of using two near-infrared wavelengths to observe the $O_2 + Hb \rightarrow O_2Hb$ reaction.

559

Project Description:

Objectives: The objectives of this project are to develop new instrumentation methods, data handling techniques and theoretical treatments for the physiochemical study of the thermodynamics, kinetics and thus the mechanisms of enzyme action in solution and in the intact cell or cell membrane. In particular, to develop the method and instruments to study, in collaboration with other laboratories, the reactions of hemoglobin with the respiratory gases both in the normal state and as modified by the changes of physical factors, small molecules, various metabolites, and genetically, such as in sickle cell anemias. The reactions of various cellular enzymes, particularly ATPase and lactate dehydrogenase, and their interactions, and control, in the cell are studied as they relate to the hemoglobin reactions in cardiology, pulmonary, and respiratory function, and circulation. Where appropriate analytical methods are developed for research and clinical application.

Methods Employed: The methods used in the investigation of the mechanisms of enzyme action are those of pre-steady state chemical kinetics and thermodynamics. Measurements of the appropriate parameters are made by developing the necessary equipment to mix solutions rapidly and follow the course of the resulting chemical reactions by optical, thermal, glass electrode, etc., detectors. In general, equipment is not available, either in the literature or commercially, for investigations in this area. Such apparatus is conceived and designed in this laboratory, together with consultants, construction being carried out wherever most appropriate; i.e., in our shops or by commercial firms, special university facilities, or at the several special research laboratories such as the Jet Populsion Laboratory. In pursuing these investigations, a wide variety of physical parameters must be studied, which leads to the need for an understanding of the underlying physical theory governing the reactions. Expert constultants and collaborators are brought in to assist in the design, analysis, and evaluation of the equipment, particularly as it applied to certain specific enzyme systems under investigation.

Major Findings:

Results of the development of instrumentation for the study of rapid biological reactions may be grouped under optical, pH, PCO_2, thermal and laser flash photolysis. The high speed flow system has been finally put into operation with the completion of a new observation cell through the cooperation of our plastics group in BEIB and the Emerson-Cummings Corp. Appropriate casting and cementing materials have been obtained. Excellent work by the glass blowing shop of BEIB has produced glass and quartz windows of the correct size and shape so that the total unit can be made without shrinkage, smooth, and of such a character that no cavitation occurs at the flow velocity of 40 meters per second. Furthermore, the system appears to be distortion free at the time of the stopping of the

solutions. The completion of experiments on the reaction of calcium with EGTA at 25^{o} and 4^{o} is of great importance to muscle physiologists and should be finished shortly.

Several coatings have been tested on various pH electrodes. At this point we are using Lycra in a 1:5 dilution. This appears to greatly protect the electrode, including a new 3 millimeter combination electrode made by Ingold which has a porous plug. The speed of the system has not been fully tested but it is faster than 100 milliseconds. By utilizing the 3 millimeter combination electrode, Lycra coating, and carbonic anhydrase bound to glass beads, a new PCO_2 electrode has been constructed with a response of 1 second for a 10% to 90% change. This is badly needed for the oxygen dissociation curve work and for continuous flow PCO_2 experiments. Should the electrode prove even faster than we believe, it might be possible to use it for stopped flow experiments.

Final fabrication of a fast 3 millisecond response time thermistor has been completed. A new thermal stopped-flow apparatus built and an initial tests performed indicating a sensitivity of .1 millidegree for the system using about 200 µl of each reagent per reaction. Hemoglobin reactions with carbon dioxide and 2-3DPG will be studied with the system both in solution and red blood cells.

The liquid dye laser system has been set up and tested and an optical system constructed and installed. A 10 Mzh Optisat is under construction to permit the reactions to be studied in whole blood, including sickle cell. Preliminary testing is underway with carboxy-hemoglobin and oxy-hemoglobin reactions.

An interesting problem in the study of the use of insulin is that of the measurement of glucose in the blood stream. A new glucose oxidase enzyme has been extracted from penicilum notatum with the assistance of the the large scale enzyme preparation system at Oak Ridge National Laboratory. 100 milliliters of this enzyme of very high activity have been obtained. By mounting the enzyme on glass beads, which in this case will be thermistors we hope to be able to monitor directly in the blood stream the level of glucose as the insulin is administered. The amount of insulin injected can be directly controlled by a measurement of glucose level. The speed of reaction of this system is in the millisecond range. The glucose oxidase can also be used in the oxygen dissociation curve apparatus, since by adding a few beads the oxygen contained in the sample can be removed utilizing the glucose already present in the blood and the addition of a small amount of glucose to ensure that all of the oxygen present is removed. This will be of particular importance in measuring the oxygen dissociation curve of sickle cell blood.

Proposed Course: Work will continue on the various systems and detectors

to bring each instrument to the point where it's usefulness to the biomedical scientist has been established. Efforts will then be made to have it available from a manufacturer.

Publications:

1. Froehlich, J. P., Sullivan, J. V., and Berger, R. L.: A Chemical Quenching Apparatus for Studying Rapid Reactions. Anal. Biochem. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01414-04 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Development of Microcalorimeters for Clinical Chemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Berger	Research Physicist	LTD NHLI
OTHERS:	N. Rehak		CP:CC
	M. Marini		Northwestern U. Med. School
	N. Davids		Pennsylvania State U.
	B. Balko		Boston, Univ.

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch, N.I.H.
Clinical Chemistry Department, Clinical Center, N.I.H.

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2¼	1¼	1

SUMMARY OF WORK (200 words or less - underline keywords)

Development of the batch microcalorimeters has been continued with major emphasis being placed on improvement of sample cells, electronics, temperature regulators and the development of a microprocessor for data processing. Several enzyme reactions are being studied with a view to clinical analysis. Because of the large amounts of enzymes needed for the required sensitivity several of the enzymes are being immobilized on glass beads so that they can be used repeatedly. A fluidic logic flow system has been developed and tested on the stopped-flow microcalorimeter. Both this unit and the syringe drive unit are operating well and the only remaining problem is obtaining rapid, thermo-equilibrium of the incoming solutions. The artifact from mixing is less than 1 microcalorie. A new combined pH-thermal titration cell has been constructed and tested. Several improvements are being made and a differential cell is also under construction. A sensitivity of 0.1 milli-degree is now routine in a 2 ml sample.

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PHS-6040
(12-75)

Project Description:

Objectives: Virtually all chemical reactions produce heat and calorimetry has long been used to investigate them. For biological use, however, high sensitivity, small volumes of reactants, and short equilibration times are needed. It is the objective of this project to develop such an instrument for use in the time range of a few seconds to 1 or 2 hours.

Methods Employed: Initial designs are constructed in this laboratory with special assistance from commercial firms in the construction of sensors; contracts are let, where warranted, for the development of a completed instrument with refinements that would tax our own facilities. The instrument is then tested in conjunction with other interested biochemical calorimetrists utilizing appropriate enzymatic and cellular reactions.

Major Findings: The introduction of microcalorimetry in clinical chemistry has proceeded reasonable well during the past year. The single and differential batch calorimeters as well as the flow calorimeter are now made commercially and six of the batch instruments are in operation. The unit operating in Clinical Chemistry has been used primarily for the study of protolytic enzymes in feces and details of this work will appear in the Clinical Chemistry report. The unit at Oxford University has continued to be used primarily on liver cell preparations and a full report on this work should be ready next year. Work continues in this laboratory on improving the performance of the present system by improving the sample cells, some improvement in the amplifier electronics and thermal regulators, and introducing a microprocessor to take and correct the data so that immediate on-line results can be obtained. Microprocessors will also be used for totally automating the flow calorimeter. A fluidic logic flow system has been constructed which permits the injection of buffer enzyme in a carefully controlled manner so as to minimize the mixing artifacts to less than 1 microcalorie. Further work is needed to bring the thermal equilibrium of the entering reactants to the same low level. The development of the thermal-pH titration apparatus has been included in this project because of the important potential for its use in antigen-antibody, coagulation and enzyme analysis work. A new cell has been built and tested and a differential model is presently under construction. A series of amplifiers have greatly improved the performance of the instrument. A new stirring system and further improvements in cell design have been done in collaboration with the electrical and mechanical engineering sections of BEIB. A microprocessor will be included in the new differential system so that thermal regulation, stirring, data collection, and correction can all be made automatically. A sensitivity of 0.1 milli-degrees or better is now readily obtainable on a 2 ml sample.

Proposed Future Research: The effectiveness of the batch microcalorimeter as an instrument suitable for routine clinical work will continue to be explored in collaboration with Clinical Chemistry and Molecular Hematology.

Additional exploration of other chemical reactions is planned, particularly in the area of triglyceride, cholesterol and antigen-antibody reactions as well as the determination of 2-3 DPG in the red cell. Considerable work is needed to solve a number of technical problems associated with high reliability and sensitivity of the stopped-flow microcalorimeter and these will be continued to be pursued. The titration calorimeter needs additional work in the development of the differential cell and this will be pursued in the coming year.

Publications:

1. Berger, R. L., Davids, N., and Panek, E.: The Design and Development of a Stopped-Flow Microcalorimeter for the Study of Enzyme Kinetics. Journess de Calorimetrie et D'Analyse Thermique, Vol. 6: pp 6.1 - 6.10, 1975.

2. Rehak, N. N., Everse, J., Kaplan, N. O., and Berger, R. L.: Determination of the Activity and Concentration of Immobilized and Soluble Enzymes by Microcalorimetry. Anal. Biochem. 70: 381-386, 1976.

585

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01415-03 LTD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Italy-U. S. Cooperative Science Program - Blood Gas Instruments - Project 78

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert L. Berger	Research Physicist	LTD NHLI
	Luigi Rossi-Bernardi		University of Milan, Italy
OTHERS:	M. Luzzana		U. of Milan, Italy
	R. Winslow		MHB NHLI

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS, NIH

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1½	½	1

SUMMARY OF WORK (200 words or less - underline keywords)

The Italian - U.S. Science Cooperative project has thoroughly tested the oxygen dissociation curve apparatus designed for both clinical and research use. Particular attention has been paid to the problems of determining the saturation from 0-2% and 98-100% to ± 2%. The rest of the curve being readily determined to this precision. A large number of normal and abnormal hemoglobin patients have been tested. Some automation of the system has been achieved through the use of a microprocessor and a totally new instrument which will be completely automated is under construction. Work has started on the laser flash photolysis system for whole blood work. A novel optical method for very accurately obtaining the concentration of various derivatives i.e., CO, O_2, met hemoglobin concentrations is also under construction.

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PHS-6040
(12-75)

Project Description:

Objectives: The total oxygen needed by a normal healthy subject is provided by the circulatory system according to the well known equation:

O_2 consumption = cardiac output x arterial-venous O_2 difference.

Since several pathological conditions can shift the oxygen dissociation curve (ODC) and thus how much oxygen can be released to the tissues, it is of considerable clinical and fundamental physio-chemical interest to be able to measure the ODC under true physiological conditions of the patient. The aim of this project is to develop instrumentation to provide a comprehensive analysis of the various chemical factors regulating the (A-V) O_2 difference or, more generally, the oxygen dissociation curve of human blood under various physiological or pathological conditions. ODC position and shape is under control of various small molecules or ions, i.e. CO_2, protons, and 2,3-DPG, etc.

Methods Employed: A systematic analysis of the complex interrelationship among several variables and their effect on the oxygen dissociation curve requires the development of a simple method to obtain oxygen dissociation curves of human blood, in vitro, under conditions closely simulating the in vivo situation of the patient.

Instruments are developed either at NIH and/or Milan, tests on pure hemoglobin are generally conducted first in Milan where a large group is currently working on the purification of hemoglobin. Testing on patient clood is then carried out in the Molecular Hematology Branch, NHLI. Close cooperation exists with the medical school hospital in Milan where on-line computer monitoring will be carried out using the membrane oxygenator system, developed in this laboratory by Dr. Kolobow.

Major Findings: The oxygen dissociation curve apparatus has been installed in the Molecular Hematology Branch and is being used daily for patients. Several important points have arisen as a result of such usage.

The large amount of data generated and its need to be analyzed and stored has resulted in our developing the appropriate computer programs using the PDP-10 MLAB lab system and recommending to MHB the purchase of the appropriate equipment to interface with the PDP-10. This is now a regular operating system. A microprocessor system has been built using an Intel 8008 microprocessor with the arithemetic unit being a Texas Instrument SR50 calculator. This system was initiated to operate the entire system, However, the SR50 has proved to be somewhat slow in making the on-line calculations and a new system based on an Intel 8080 microprocessor is under construction. The SR50 system did give us an opportunity of working out all the mechanical and electrical details as well as the computer programs which would be needed. Several problems have occured with the oxygen dissociation curve

apparatus and a two state redesign of the instrument is under way with the assistance of the Mechanical and Electrical Engineering Sections of BEIB. The present unit was made by modifying an IL blood gas apparatus. The interface with the PO_2 and PCO_2 electrodes trap bubbles which are sources of air. Further a magnetic stirrer is used which tears the PCO_2 electrode about every 4th run which of course, destroys that run. A new unit has been designed and construction almost completed by BEIB which will correct this problem. The PCO_2 detectors available are very slow and their response to a 67% change in PCO_2 is at best 20 seconds, whereas the oxygen detector is up 90% in two seconds. By using a new combination electrode from Ingold, Zurich, coating it with Lycra, and using carbonic anhydrase bound to glass beads in a TEA buffer pH 7.8 and the standard 3 mil silastic Kolobow membrane material a 90% rise in less than 1 second was obtained with a PCO_2 change from 0-10% CO_2. Extensive testing will be done on this new electrode but we feel it is a major improvement in PCO_2 measurements. The second design stage of the new oxygen dissociation curve will incorporate both this smaller PCO_2 electrode and smaller PO_2 electrode as well as the ability to monitor several wavelengths using a modification of our Optisat oxygen saturation monitor so that an accurate determination of the various hemoglobin components can be made on the whole blood. It is hoped that a new method of degassing the blood will reduce the time from the present 20-30 minutes to 2 minutes. The entire handling of the system will be done under the control and check of the microporcessor, so that the technician will simply have to add the blood.

Publications:

1. Rossi-Bernardi, L., Luzzana, M., Samaja, M., Davi, M., DaRiva-Ricci, D., Minoli, J., Seaton, B., and Berger, R. L.: Continuous Determination of the Oxygen Dissociation Curve for Whole Blood. Clin. Chem. Vol. 21. No. 12, 1975.

2. Rossi-Bernardi, L., Luzzana, M., Samaja, M., Rossi, F., Perrella, M., and Berger, R. L.: The Functional Properties of Sickle Cell Blood. FEBS Letters, Vol. 59, No. 1, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01417-01 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Capillary Flow Method for Determination of Cell Osmotic Fragility (Part I)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Ito	Visiting Scientist	LTD NHLI
	P. Carmeci	Electronic Engineer	LTD NHLI
	R. Steele	Physical Scientist	LTD NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

A simple and accurate micromethod for the determination of cell osmotic fragility is introduced. The method uses the Poiseuille's parabolic flow pattern together with the gravity to retain cells in a long capillary tube while a solution with decreasing osmolarity is passed through the tube for hemolysis. Hemoglobin released from the cells is quickly removed by the axial flow pattern and monitored with a spectrophotometer for recording the hemolysis curve. A comparision is made with present methods and test results from normal and abnormal individuals are reported. Advantages are simplicity, reproducibility, and accuracy with 1 to 2 ul sample doses and versatility for possible automation in clinical laboratory as well as a research tool for various types of cell studies.

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Objectives: Development of a simple accurate micromethod for determination of cell osmotic fragility.

Method Employed and Major Findings:

A. Principle: A saline solution with a desired osmotic gradient is prepared by means of a gradient mixer and passed through a standing fine coiled tube. Due to the Poiseuille's flow pattern together with the gravitational effect, erythrocytes introduced into the coil move through the coil at a rate much slower than that of the gradient solution. Thus, the cells are exposed to a gradual decrease in osmolarity for hemolysis. Hemoglobin released from cells is then quickly removed by the flowing stream and detected through a spectrophotometer at 547 nm for recording hemolysis curve.

B. Apparatus: The basic elements of the system used are a gradient mixer to make the gradient, a pump to provide the flow, a coil to provide the retention of the red blood cells and a detector and recorder to monitor the hemolysis of the cells.

The gradient mixer is an LKB Ultrograd 11300 with a pair of valves and mixing set to deliver a gradient of .85% to 0% NaCl over a period of 30 minutes. The gradient solution is withdrawn from the mixer by a Technicon Proportioning Pump through a length of .065" I.D. Autoanalyzer pump tubing to a bubble trap. A smaller diameter length of .015" I.D. tubing goes back through the pump to introduce a bubble-free gradient solution into the coiled column. At the exit to this second pumping stage is a threaded connection with a well in which to inject the sample. The sample and gradient solution pass through the coil and thence to an LKB Uvicord II detector and recorder. The cell used in the Uvicord detector was a 1.8 mm I.D. flow cell with a 547 nm interference filter for detection of hemoglobin.

The coil is a piece of .85 mm I.D. Teflon tubing 140 cm long with a capacity of .8 ml. Larger diameter tubing gave insufficient washing of the blood cells with the flowing saline solution, this manifesting itself in unnecessary broadening of the hemolysis peak. In smaller diameter tubing mixing was too violent and carryover of the cells took place. The optimum length of the coil was determined by the flow rate (6.7 ml/hr) and the gradient rate (.85-0% NaCl for 30 min) selected.

C. Experimental Procedure: The following gradient solution preparation is based upon the method of Parpart and co-workers.

A stock solution of buffered sodium chloride osmotically equivalent to 10% NaCl is made as follows: NaCl 90 gms, Na_2HPO_4 13.65 gms, and $NaH_2PO_4 \cdot H_2O$ 2.15 gms are dissolved in distilled water and final volume adjusted to

1 liter. The starting solution being equivalent to .85% NaCl is prepared by diluting 85 ml of the stock solution with distilled water to bring the final volume to 1 liter. This final solution had a pH of 7.4. Distilled water is used as the ending solution. The linear gradient between starting and ending solutions is obtained with the LKB Ultrograd with a 30 min. time scan.

The tests were performed as follows: The sample port was opened and the pump run for about 1 min. on rapid wash cycle so that the pump tubing was flushed with the starting solution. A syringe containing some starting solution (about 3 cc) was used to flush through the detector and coil from the elution end to the sample port where excess was removed with a vacuum probe. The sample was then introduced into the sample port and the port closed. The pump and gradient mixer were then started simultaneously with the recorder.

Comparative tests were made using the Parpart method. Incubation tests were performed on undiluted blood incubated for 24 hours at 37°C.

Undiluted blood treated with heparin or EDTA were used as samples and injected with a micropipette or Hamilton 10 µl syringe. In these studies both heparin and EDTA treated blood gave the same results. This is probably due to the fact that these anti-coagulants are immediately washed away by the buffered gradient solution.

A calibration of % NaCl vs. time was made on the recorder using a pre-calibrated Chromatronix conductivity meter.

D. Results: A series of studies were made to evaluate the capability of the capillary flow method. Optimum sample size ranges 1 to 3 ul where the shape and peak location of the hemolysis curve remain similar. Using 2 ul sample dose from the same blood sample, a high reproducibility was demonstrated on 5 successive runs. Comparison studies were made on 20 normal and abnormal samples. Our values (peak location) highly correlated to the 50% hemolysis point of the Parpart method in both fresh and incubated samples. Several abnormal blood samples were also examined with interesting results. The Thalassemia major exhibits low osmotic fragility in fresh samples which further decreased after incubation. The sickle cell anemia showed low osmotic fragility in both fresh and incubated samples but in this case the whole population became more fragile upon incubation similar to the normal sample. One abnormal case with diagnosis unknown revealed that although the fresh blood sample is close to normal the incubated sample showed two distinct cell populations, one of which decreases in osmotic fragility as the other increases, while the 50% hemolysis point determined by the Parpart method was within the normal range.

Significance to Biomedical Research and the Program of the Institute:

The capillary flow method provides the following advantages over the conventional methods. 1) The technique is simple. 2) Sample size is small and no sample preparation is necessary. 3) The accuracy and reproducibility is high. 4) The output is in a nonintegrated form. 5) The system is very amenable to the changing of parameters, such as shape and slope of the gradient, flow rate, temperature, monitoring wavelength, etc. for the study on osmotic fragility in other types of cells. 6) The system is also adaptable to automation for many samples simultaneously and/or successively. 7) The eluent can be fractionated for further analysis.

Proposed Course:

1. Refinement of the system (Reported in Part II)
2. Studies on blood samples from newborn babies, infants, and stored blood.
3. Studes on other types of cells including peripheral leukocytes, bone marrow cells, etc.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01418-01 LTD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Fast Responding Oxygen Electrode

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	G. Vurek	Senior Investigator	LTD NHLI
OTHER:	J. Danek	Guest Worker	Air Shields, Inc.

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with the design and construction of an O_2 electrode with high electrical output, low O_2 consumption per unit area, and fast response. We are studying the application of silver mesh as cathode material on the performance of an electrode system with the above properties. The silver mesh should reduce the oxygen consumption per unit area and avoid the usual problem of variable electrolyte thickness between the cathode and protective membrane.

PHS-6040
(12-75)

Objectives: The purpose of this study is to develop an oxygen sensing electrode which has a rapid (seconds) response time, is covered with a protective membrane, and uses a small amount of oxygen from the sample. The linearity, stability, and freedom from pressure and stirrring effects will be explored.

Methods Employed: Heretofore Clark-type oxygen electrodes have used a membrane to separate the electrode from unwanted electrochemically active species in the sample. There is also a layer of electrolyte between the electrode and membrane. Either the electrode/membrane combination or the membrane alone is usually designed to be the principal diffusion barrier. If this is the case, most of the oxygen gradient can be kept under the manufacturer's control and stirring artifact is small. Difficulties are that if fast response is needed high permeability membranes must be used so that for reasonable freedom from artifact, very small electrodes are needed which means very small currents are produced. Moreover the electrolyte layer thickness may vary with pressure, changing the response.

Our electrode incorporates the protective membrane with a porous electrode. When oxygen diffuses through the membrane and reaches the metal on the back, it is reduced electrochemically. Thus, there is no uncertain electrolyte path. The effective permeability is determined by the pore structure of the metal. Therefore, fast-high permeability membranes can be used because the metal provides the principle barrier.

Major Findings: Initial studies were made with silicone rubber coated on one side with carbon black. The carbon film was used as the cathode and a silver / silver chloride electrode was used as the anode. In some tests, a zinc sheet was used as the anode. These tests demonstrated that oxygen could be reduced at a carbon cathode. Transient response tests showed that response of the electrode was complete in less than 2 seconds. Similar results were obtained with 300 line/inch (75 μm spacing) silver mesh covered with silicone rubber. However, both these electrodes had high permeability and were not appropriate for stirring effect tests. Low porosity silver mesh (about 0.2% hole area) is on hand and tests are being prepared.

Significance to Biomedical Research and the Program of the Institute: Because conventional oxygen electrodes consume substantial current per unit area, they may be sensitive to stirring effects. The new design should be a simple way to reduce the stirring effects and yet permit rapid response. This is useful for measuring the PO_2 in thin films of fluid or on surfaces of tissues.

Proposed Course: The porous silver electrode will be evaluated for response time and the effect of stirring and pressure. In addition, its electrochemical properties will be measured.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01419-01 LTD

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Transcutaneous Oxygen Measurement

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. G. Vurek	Senior Investigator	LTD NHLI
OTHERS:	J. Danek	Guest Worker	Air Shields, Inc.
	T. Kolobow	Chief, Section on Pulmonary and Cardiac Assist Devices	LTD NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.4	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

Continuous non-sampling measurement of arterial PO_2 is desirable in some types of physiological investigations and some acute clinical situations. If the skin can be adequately perfused, oxygen from arterial blood will reach the surface in measurable quantities. This transcutaneous oxygen measurement coupled with a measure of relative skin blood flow, may provide a good measure of arterial PO_2. This program is designed to develop a suitable oxygen electrode and perfusion measuring instrument for transcutaneous use.

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Objectives: It is the objective of this project to obtain a reliable indication of arterial blood PO_2 by measuring oxygen diffusing to the surface of the skin. The accuracy of this sort of measurement depends on the availability of arterialized blood at the outer layers of the skin so that the measurement is of limited value during shock, hypothermia, or other situations where skin blood flow may be impaired. Nevertheless, there are situations where non-sampling continuous measurements are desirable and where adequate skin blood flow can be obtained with local heating.

Methods Employed: The basic instrument consists of a sensitive oxygen electrode coupled with a heater that can be used to measure thermal conductivity of the skin under the electrode. Oxygen normally diffuses from the atmosphere into the skin so that without adequate blood flow from deeper layers, the PO_2 of skin protected from atmospheric O_2 is less than 5 Torr. Even with good perfusion oxygen must diffuse through the non-vascular layers of skin so that the probe must not consume too much oxygen which could cause a significant systematic error. The heater circuit senses the amount of power required to maintain a given temperature. Much of the power applied is carried from the electrode by the skin, so that changes in skin blood flow produce changes in thermal conductivity and influence the heater power requirements. The combination of the two measurements can give an indication of arterial PO_2 and whether the indicated value is reliable.

Major Findings: The work to date has been centered around electrode design. The results of this work is summarized below.

To obtain an accurate measure of the arterial PO_2 the electrode must have a permeability at least 1/10 that of the skin. The skin has a permeability equivalent to about 200 μm of water. In order to obtain a reasonably rapid response, eg., 63% of final value in 15 sec., the electrode membrane must be thin. These requirements restrict the membrane material choice to those such as polyester (Mylar), chlorinated-fluorocarbons (Kel-F, ACLAR), polyamids (Nylon), and polyethersulfone. In addition to the oxygen properties, a low water vapor permeability is desired to keep the electrode from drying out when it's not in use. The best material we have found so far is an Allied Chemical Co. product, ACLAR-33. It can be made 8μ thick, has low oxygen permeability and very low water permeability. It is also resistent to heat and many organic solvents as well as aqueous solutions. We will use the ACLAR membrane.

Cathode materials have also been considered. Although platinum and gold have been used in most oxygen measuring electrodes, oxygen can be reduced at a silver surface. Silver offers the advantage that the entire electrode system can be made of silver; the anode can be Ag-AgCl. This approach reduces the number of dissimilar metals in the system and prevents plating of anode silver on a non-silver cathode. The silver cathode has a high

thermal conductivity so that heater power is transmitted through the cathode to the skin. A silver-silver system covered with an ACLAR-33 membrane has been tested for 5 weeks and has not demonstrated more than 10% drift (except for ambient temperature effects). We feel the use of the silver cathode is justified.

Skin blood flow contributes to the total skin thermal conductivity. On the basis of measurements using the CO_2 probe and on calculations based on literature data, we expect to have to measure conductivities or changes on the order of a few milliwatts/cm^2-degree.

Significance to Biomedical Research and the Program of the Institute:

Transcutaneous measurement of arterial PO_2 offers a non-invasive monitoring technique. It can be useful to monitor neonates and adults requiring respiratory assistance as well as exercise test subjects. Its value lies in the convenience and non-invasiveness. It is limited to those subjects with good peripheral circulation.

Proposed Course: We propose to construct electrodes suitable for transcutaneous PO_2 measurement and evaluate their response in vitro. In addition we will investigate the effect of prolonged local hyperthermia on skin blood flow using the laser doppler skin blood flowmeter. Measure ments on sheep undergoing extracorporeal circulation may also be made.

Publications: none.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01421-01 LTD

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Critical Care Blood Gas, pH and Electrolyte Analysis Methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. E. Steele Physical Scientist LTD NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

SUMMARY OF WORK (200 words or less - underline keywords)

The basic simplicity of ion-specific electrodes makes them attractive for the measurement of certain electrolytes in automated blood analysis instrumentation. Steele, et. al (Proc. San Diego Biomed. Sympos. 14: 469-478, 1975) have used a programmable calculator system to overcome many of the well-known problems of ion-specific electrodes - interferences, drifts, and liquid junction potential changes. They have also shown that such a calculator system makes it possible to quickly convert the activities which ion-specific electrdes measure into the concentration units which are widely employed clinically for serum electrolytes. This conversion, is based on factors (serum water content, activity coefficients and ion binding by proteins) which may vary significantly in critically ill patients. In order to further establish the advantages and limitations of ion-specific electrodes for the determination of serm electrolytes of critically ill patients, a programmable calculator and data acquisition system with capabilities greater than that of Steele, et al. has been assembled, programmed and put into operation. The construction of a pH electrode and the design of a total CO_2 measurement device have been undertaken with the objectives of great simplicity and small sample size.

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(12-75)

Objectives:

(1) To further study the relationships between the activities of serum electrolytes measured by ion-specific electrodes and the concentration of these electrolytes in the units now used clinically. The factors involved in this relationship (serum water content, activity coefficients and ion binding to proteins) are most likely to be changed significantly in critical illness and diseases states were electrolyte content, pH or serum proteins are greatly changed.

(2) To better define the interferences suffered by solid state Cl^- and glass K^+ ion-specific electrodes so that these electrodes may be used to check on the performance of liquid membrane Cl^- and K^+ electrodes in cases where the interfering materials are also being measured. (Protein interferes with AgCl Cl^- electrodes in a pH dependent fashion. Na^+ interferes with the glass K^+ electrode in a time dependent fashion.) The use of redundant sensors can greatly increase the reliability of highly automated instrumentation.

(3) To evaluate changes in liquid junction potentials caused by serum proteins. The principal investigator has found that the simultaneous use of ion-specific electrodes sensing both + and - ions (i.e. Na^+ and Cl^-) is of considerable help in evaluating the troublesome liquid junction potentials.

(4) To construct glass pH and K^+ electrodes in a flow-through configuration which is simple to make and requires a small sample (i.e. 1 microliter). An additional objective is to evaluate a pH electrode design in which it is feasible to reduce the sensing volume to about 10 nanoliters for use in kidney tubule perfusion studies by Dr. Burg's group in NHLI-LKEM.

(5) To simplify the differential conductance CO_2 method of Steele et al for total CO_2 of blood serum so that the requirements for cylinders of carrier gas and solution pump are eliminated. In this form the method should be very much simpler than the Van Slyke technique as well as requiring only 1 microliter or less of sample.

Method Employed and Major Findings:

Exceptionally good results can be obtained from ion-specific electrodes by using a programmable calculator system for data acquisition, storage and analysis as shown by Steel et. al (Proc. San Diego Biomed. Sympos. 14: 469-478, 1975). Such a data handling system makes it feasible to run standards frequently to deal with drifts and sensitivity (slope) changes. Corrections for interferences and liquid junction potential changes become easy and rapid. Readings can be taken automatically on the basis of equilibrium criteria which are objective and reproducible. In addition, such a calculator system makes it possible to convert the electrolyte activities measured by the ion-specific electrodes into concentration

units such as determined by flame photometry and chloride titration. This conversion and subsequent comparison of the results with conventional measurements is important in establishing the validity and clinical meaning of ion-specific electrode determinations particularly for critically ill patients. The conversion is primarily based on serum water content, activity coefficients and ion binding to proteins. For many types of serum, the calculator enables one to quickly obtain adequately accurate estimates of these factors from two simple determinations: refractive index and conductivity (as shown by Steele et al).

In order to take advantage of these capabilities, such a programmable calculator system has been assembled, programmed and put into operation. The calculator is a Hewlett Packard Model 9830A with printer and plotter. The use of an interface bus gives great flexibility in connecting the digital voltmeter, timing generator, relay actuator and digital to analog converter for data acquisition and experimental control. Programming of the devices on the interface bus is remarkably convenient. During the few months that the system has been in operation, its major use has been for the data handling in the Capillary Flow Method for Determination of Cell Osmotic Fragility project in this laboratory. That use will be described separately. That project and this blood analysis project have a number of common interests which make this digression logical.

A flow through pH electrode with a 3 microliter sensing volume has been constructed from Corning 0150 glass having 0.040" O.D. and 0.020" I.D. The contact to the outside glass surface (sample is inside) has been made with solid AgCl with a fine Ag wire embedded in it. This was done by rolling the glass in molten AgCl before and after winding on the Ag wire. The structure was insulated by rolling it in molten polyethylene. This method of contacting the surface of the glass opposite the sample (rather than the usual vessel containing 0.1 N HCl and a Ag-AgCl electrode) was selected because of its simplicity and ruggedness. The electrode has a resistance of 5×10^9 ohms (very near the value calculated from the volume resistivity given by Corning for this glass) and a slope at 25°C of about 55 millivolts per pH unit (59 millivolts being the theoretical value). No significant change in these characteristics was seen as a function of the time allowed for hydration. This maybe because the glass was heated only to the melting point of AgCl (455°C). The glass was used in the thickness sold by Corning so as to avoid changes due to heating and to have a rugged electrode. This thickness is greater than usual for pH electrodes and thus requires investigation.

Significance to Biomedical Research and the Program of the Institute:

Since ion-specific electrodes are being used more and more frequently both in biomedical research and in automated instrumentation for clinical determinations of electrolytes, it is important that the advantages and

limitations of these electrodes be well understood. A major objective of this project is to advance this understanding and to develop methods of overcoming the limitations of these electrodes particularly in regard to critically ill patients. The project is also expected to yield sensor designs with small sample requirements and redundant capabilities which will permit exceptional accuracy, reliability and speed in the determination of blood gases, pH and electrolytes. The rapid availability of such determinations should make it possible for the physician to better follow trends in critically ill patients and to develop improved methods of care for these patients.

Proposed Course:

(1) Further study the relationship between activities and concentrations of electrolytes in abnormal patient serum.

(2) Develop redundant methods for Cl^- and K^+ measurements.

(3) Determine the effects of serum proteins on liquid junction potentials as a function of pH.

(4) Develop a simple and self-contained method to determine the total CO_2 of microliter serum samples.

(5) Develop a flow through pH electrode with transparent walls and a sensing volume of about 10 nanoliters for use by Dr. Burg's group, NHLI-LKEM.

Publications: None

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ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart and Lung Institute
July 1, 1975 through June 30, 1976

The experimental interests of the Cardiology Branch developed over the past few years have continued. These relate to the pathogenesis, pathophysiology and treatment of coronary artery disease; the ultrastructural and molecular mechanisms responsible for normal and impaired contractile function of the heart; development of the diagnostic and investigational capabilities of echocardiography; and the application of multidisciplinary techniques to define the determinants of irreversible heart failure in patients with valvular disease and how such information can be used to determine optimal time for surgical intervention.

CORONARY ARTERY DISEASE

Pharmacologic Treatment of Acute Myocardial Infarction (AMI)

In the past few years, we showed that treatment with nitroglycerin (NTG) following coronary artery occlusion in dogs diminishes infarct size and reduces the incidence of VF occurring spontaneously during AMI. These actions are potentiated when a vasoconstrictor is administered to abolish the NTG-induced fall in arterial pressure and reflex tachycardia.

With radioactive microspheres, we found that NTG reduces ischemic damage not only by reducing myocardial oxygen demand (MVO_2), but also by increasing collateral blood flow. However, if heart rate increases excessively, collateral flow may be compromised and MVO_2 increased, thereby attenuating NTG's salutary effects. Methoxamine potentiates the beneficial effects of NTG by eliminating NTG-induced tachycardia (thereby reducing MVO_2 and increasing collateral flow) and maintaining coronary perfusion pressure. Additional studies determined that the acute increase in collateral function induced by a 1 hr. infusion of NTG and methoxamine is sustained for as long as 4 hrs. after discontinuation of drug. Hence, short-term treatment with NTG and methoxamine may result in a sustained decrease in myocardial injury following acute coronary occlusion.

We have completed our initial evaluation of this form of therapy in patients with AMI. NTG alone caused no consistent decrease in ischemic injury in patients without LV failure. However, when phenylephrine was added to reverse the blood pressure lowering and heart rate speeding effect of NTG, improvement in myocardial ischemia occurred uniformly. In contrast, NTG alone significantly improved ischemic injury in all patients with failure. Additional studies will assess the efficacy of prolonged cutaneously applied NTG on ischemic injury, arrhythmias, and LV function.

Other vasodilators, such as nitroprusside (NP), are used extensively in patients with AMI. These agents improve hemodynamic function. However, no information is available relating to the effects of NP on myocardial ischemia. Hence, we evaluated the comparative actions of NTG and NP on coronary collateral function in dogs. Although both NTG and NP reduced collateral resistance, NP was a

more potent dilator of coronary and systemic arterioles. Hence, the relative dilator potencies of these agents vary in different vascular beds -- for a given reduction in systemic arterial pressure, NP is less effective than NTG in increasing collateral function. Moreover, NP's greater capacity to dilate coronary arterioles may be deleterious since it may predispose to a coronary steal, characterized by redistribution of flow from ischemic myocardium (supplied by vessels maximally dilated by ischemia) to nonischemic myocardium (supplied by nondilated vessels that could respond to NP). This hypothesis was confirmed by comparing the effects of NTG and NP on ischemia during acute coronary occlusion in dogs: while NTG decreased myocardial ischemia NP increased it.

Determinants of Collateral Blood Flow and Ischemic Injury During AMI

We next determined the influence of hemodynamic changes on ischemia and on collateral flow. Changes in collateral flow correlated inversely with changes in heart rate and directly with changes in blood pressure. The absolute degree of ischemic damage (ST segment elevation) was dependent on the balance between oxygen supply (collateral flow), oxygen demand (assessed by blood flow to normal muscle) and heart rate. Similarly, ischemia could be reduced by increasing collateral flow or decreasing oxygen demand. Thus, for the first time it has become possible to express quantitatively in the form of regression equations the relations between ischemic injury and myocardial oxygen supply and demand.

$\%\Delta CBF = 2.04\ (\pm.93)\Delta AP - 0.58\ (\pm.28)\Delta HR + 21.5$ ($R=.66$; $p<0.01$)
$ST = -12.66\ (\pm.97)\ CBF + 3.69\ (\pm.74)\ nMBF + 0.04\ (\pm 0.02)\ HR + 5.27$ ($R=0.9$; $p<0.001$)
$\%\Delta ST = 0.24\ (\pm.10)\ \%\Delta nMBF - 0.19\ (\pm 0.06)\ \%\Delta CBF - 15.4$ ($R=.84$; $p<0.001$)
Where $\%\Delta$ = percentage change; ΔAP = change in arterial pressure (mm Hg); ΔHR = change in heart rate; CBF = collateral blood flow (ml/min/g); nMBF = normal zone blood flow (ml/min/g); ST = ST segment elevation (MV); R = coefficient of multiple regression

Characterization and Modification of Reperfusion Damage

During the course of the above studies, we observed a phenomemon we refer to as "reperfusion damage". This we defined as the damage sustained by the myocardium as a direct result of restoration of blood flow after a period of coronary artery occlusion. Additional studies disclosed that reperfusion after 5-10 min of occlusion is associated with an almost complete recovery of contractile function over the first minute of reperfusion. However, deterioration occurs over the next 15 min and persists for several hours. This phenomenon of reperfusion damage appears to be distinct from ischemic damage. These results suggest that reperfusion damage may contribute to the low output syndrome that occurs in some patients early after open heart surgery since 1) during cardiopulmonary bypass periods often elapse during which coronary arteries are not perfused and 2) this perfusion deficit is followed by reperfusion. We therefore have initiated studies to determine the mechanisms responsible for reperfusion damage and whether the damage can be reduced by pharmacologic agents. Reperfusion damage is 1) not reduced by agents that reduce ischemic damage (NTG/methoxamine, propranolol, verapamil, mannitol and

hydrocortisone), 2) increased by agents that increase movement of calcium into cells (calcium ionophores), 3) reduced by ruthenium red, an agent that blocks calcium entry into cells, and 4) reduced by glucose infusion (perhaps by accelerating glycolysis).

NHLI Type II Coronary Intervention Study

The progress report of this study, designed to determine whether lowering LDL cholesterol in pts with premature CAD and Type II hypercholesterolemia will retard the progression of CAD, is detailed in another section of the Annual Report.

Prospective Study on the Natural History of Patients with Coronary Artery Disease With Only Mild to Moderate Functional Disability

Retrospective studies on the natural history of CAD have determined that mortality rate can be predicted by the number of diseased coronary vessels and the presence and magnitude of ventricular dysfunction. Using such data, "prophylactic" coronary bypass operation is being recommended. However, these studies are based on data obtained largely from severely symptomatic pts and may not accurately reflect long-term prognosis in the pt with minimal symptoms. Therefore, pts with only mild to moderate functional disability are being studied by cardiac catheterization, exercise testing, 24-hour ECG tape monitoring, etc. Attempts will be made to determine prognostic indices. If high and low risk subgroups can be identified, then more rational decisions can be made regarding which pt should be considered a candidate for "prophylactic" operation. To date, 35 pts have been admitted to the study and the average follow-up is six months. There has been one death and two pts have become more symptomatic necessitating operation.

ECHOCARDIOGRAPHIC STUDIES

Asymmetric Septal Hypertrophy, or ASH

By employing echocardiographic techniques, in the past few years we have considerably increased our understanding of the disease spectrum embracing IHSS. Of note, it was recognized that LV outflow obstruction is only an occasional manifestation of the disease. The basic abnormality is a cardiomyopathy characterized by an asymmetrically hypertrophied heart that contains bizarre maloriented myocardial cells, most consistently in the ventricular septum. We also showed the disease is a genetic abnormality transmitted as an autosomal dominant trait with a high degree of penetrance, and found that the incidence of sudden death in children and young adults with the disease was surprisingly high, averaging about 4% per year.

This past year we documented sudden death to occur in otherwise healthy asymptomatic individuals who, only at necropsy, are found to have ASH. We have been particularly impressed with the possibility that ASH may be one of the causes of sudden unexpected death in athletes. We also have evidence indicating that more malignant forms of the disease may be clustered in specific families. To further elucidate the mechanisms of sudden death, we have begun

studying the conduction pathways of pts with ASH by HIS bundle electrocardiography. Our preliminary studies have revealed some pts with rapid AV conduction times and short recovery periods. If AF were to occur in this setting, (as it often does in ASH), the rhythm could evolve into ventricular tachycardia, VF, and death.

Although a disproportionately thickened ventricular septum is the characteristic marker of genetically transmitted ASH, we have found by echo (171 pts) and at necropsy (107 pts) that disproportionate septal hypertrophy (DSH) can occur in pts with other underlying cardiac lesions. Hence, DSH was present commonly (20%) in pts with pulmonary stenosis, and with primary pulmonary hypertension. It also occurred, but less commonly (10%) in pts with Eisenmenger syndrome, aortic valve disease, mitral valve disease, CAD, and systemic hypertension. It was not present in pts with atrial or ventricular septal defects and left to right shunt. In patients with RV overload, prevalence of DSH correlated with increasing RV pressure. Absence of disorganized cardiac muscle cells in the septum of pts with DSH and rare occurrence of DSH in relatives suggests that septal thickening in these conditions is not usually a manifestation of genetically transmitted ASH.

Echocardiographic Evaluation of Hypertensive Patients

Collaborative studies designed to assess the effects of hypertension on the heart are in progress. In collaboration with the N.Y. Hospital-Cornell Medical Center, we studied, by echocardiography, 275 pts with hypertension. Eighty-two percent had at least one abnormality on echo with 53% having concentric LV hypertrophy, 67% dilated left atria, 13% dilated aortic root, and 4% dilated LV. Less than 5% of pts had ASH. The majority of pts had no cardiac symptoms, normal ECG's and x-rays. Correlations between echo findings and metabolic abnormalities are in progress. These pts will be followed to determine whether any of the above echo findings provide prognostic information.

Echocardiographic Assessment of Cardiomyopathies

We are continuing our echocardiographic studies of cardiomyopathy. Pts can be classified into three categories: dilated cardiomyopathy, concentric hypertrophy, and ASH. The secondary cardiomyopathies (alcoholic, amyloidosis, hypereosinophilia, hemochromatosis, etc.) fall into one of the first two categories. The third is a specific genetically determined disease.

This past year we have concentrated on two types of cardiomyopathy, that due to alcoholism and that due to hemochromatosis. Our data suggest that wall thickening and mildly reduced systolic function can be detected by echo in heavy drinkers who have not as yet developed cardiac dilatation and failure. In the second study, pts with severe congenital anemia requiring chronic transfusion were evaluated. We found in these pts with iron overload 1) cardiac abnormalities can be detected by echo prior to clinical decompensation, 2) serial study may permit detection of disease progression, and 3) marked reduction in ejection fraction is an ominous prognostic sign.

Real-Time Two-Dimensional Echocardiography

Over the past few years, we have developed a sector-scanner which produces real-time two-dimensional visualization of internal cardiac structures. This provides a powerful tool for diagnosing anatomic relations of complex congenital abnormalities. By sequential scanning of the heart through planes perpendicular to the long axis of the LV, we found that tetralogy of Fallot, complete transposition of the great vessels, and double outlet RV can be reliably diagnosed in infants, children and adults. We also are coupling Dopler techniques with the sector-scanner to detect blood flow patterns in various portions of the heart and great vessels. Such a technique may eventually allow detection noninvasively of elevated pulmonary vascular resistance and of the relative magnitudes of intracardiac shunts.

VALVULAR HEART DISEASE

Elucidation of the Determinants of Irreversible Myocardial Failure

We previously completed a retrospective study of long-term survival in pts operated on for aortic regurgitation (AR). Although absolute heart size preoperatively did not influence long-term postoperative survival, change in heart size as assessed over the first 4-6 months following operation did. Thus, 85% of pts operated upon for AR whose cardiothoracic ratios decreased survived 6 years, as compared to only 43% of pts whose heart size was unchanged or increased. This prompted a prospective multidisciplinary study to define 1) whether a particular grouping of preoperative functional derangements leads to prohibitive operative risks, and 2) what type of derangements can be reversed or improved by operation. Evaluation of myocardial function includes calculation of ventricular volumes, EF, exercise testing, etc. Biopsies also are obtained at operation for microscopic analysis. Preliminary analyses of the pre- and postoperative data in pts with isolated AR has revealed that: 1) operation does not improve basal ventricular function, 2) LV volume and mass are more likely to return toward normal in pts with normal pre-op EF, 3) long-term results are good in pts with a high pre-op EF, poor in pts with a low pre-op EF. These findings are now being applied to patients followed in our OPD to determine whether echo assessment of changes in LV volume and EF provides a more sensitive means than traditional clinical parameters to judge optimal time for operative intervention. We also are beginning to employ scintigraphy in assessment of LV function in these pts. By labelling the blood pool with Technetium tagged serum albumin, we are able to obtain an iv radionuclide angiogram, which permits sophisticated analyses of LV function both at rest and during exercise. Preliminary results with this technique have demonstrated that pts with normal indices of LV function at rest may develop marked abnormalities during exercise.

MOLECULAR MECHANISMS RESPONSIBLE FOR
CARDIAC CONTRACTION AND CELLULAR PROLIFERATION

The Section on Molecular Cardiology has conducted research in the following areas: 1) cardiac contractile proteins - phosphorylation of cardiac contractile proteins and studies on the maturation of chick cardiac myosin. 2) Smooth muscle contractile proteins - the effect of phosphorylation of smooth muscle myosin on the interaction of actin and myosin. 3) Contractile proteins from non-muscle cells - the phosphorylation of contractile proteins in platelets, atrocytic-neuroglial cells as well as prefusion rat myoblasts and tumor cells.

1) Cardiac contractile proteins: In order to study the possible relation between cardiac contraction and phosphorylation of cardiac contractile proteins, we developed an isometric myograph suitable for prolonged (6 hr) incubation of papillary muscles in the presence of ^{32}Pi. We have studied the phosphorylation of myosin and the myosin associated M-line protein in the presence and absence of norepinephrine. Our findings to date indicate that norepinephrine increases the phosphorylation of both the 19,000 dalton myosin light chain as well as the 165,000 dalton M-line protein.

2) Smooth muscle contractile proteins: Smooth muscle myosin has been purified from guinea pig vas deferans. This myosin has a molecular weight of 460,000 and is composed of two heavy chains (200,000) and two different light chains (20,000 and 16,000 daltons). The 20,000 dalton light chain is phosphorylated by a kinase which has been purified from the smooth muscle myosin by Sepharose 4B chromatography. This kinase is similar to that found in skeletal muscle and, in contrast to the one found in platelets, requires Ca^{2+} for activity. Evidence for the existence of a phosphatase that rapidly dephosphorylates smooth muscle myosin was also obtained.

Preliminary experiments indicate that the phosphorylation-dephosphorylation of smooth muscle myosin plays an important role in controlling the actin-activated myosin ATPase activity.

3) Contractile proteins from non-muscle cells: Actin and myosin are now known to be present in most, if not all, eukaryotic cells. Studies from this laboratory established that phosphorylation of the 20,000 dalton light chain of platelet myosin results in a 5-8 fold increase in the actin-activated myosin ATPase activity. Dephosphorylation results in a decrease in this enzymatic activity. These results suggest that phosphorylation acts as a switch that controls actin-myosin interaction. To further understand this regulatory mechanism, we have continued our studies on a) platelets, b) phosphorylation of other non-muscle as well as muscle myosin.

a) Platelets: Myosin, which was mostly unphosphorylated, was separated from its loosely bound kinase by Sepharose 4B gel filtration. When this partially purified kinase was added back to the myosin in the presence of Mg^{2+} and ATP approximately 0.9 moles of Pi were incorporated per mole of 20,000 dalton light chain. This increase in phosphorylation was accompanied by a seven-fold increase in the actin-activated ATPase activity.

b) Phosphorylation of other non-muscle and muscle proteins: Recent studies suggest that the myosin kinase originally found in human platelets is present in many, if not all non-muscle cells. Studies utilizing mouse astrocytic neuroglial cells, prefusion rat myoblasts and human rhabdomyosarcoma cells revealed that each of these cells contain a myosin light chain kinase similar to the kinase isolated from platelets. These non-muscle kinases differed from the kinase isolated from skeletal muscle in substrate specificity and indepen-dece of Ca^{2+} for activity.

The presence of the cytoplasmic (non-muscle) kinase in both prefusion myo-blasts and rhabdomyosarcoma cells is of particular note. Both cells are capable of undergoing cell division in contrast to post-fusion myoblasts. Preliminary experiments with post-fusion cells indicate that these cells differ from prefusion myoblasts in two respects. a) they have a different complement of myosin light chains, b) the kinase is of the type found in skeletal muscle rather than in non-muscle cells.

Future experiments with the kinase-phosphatase system in non-muscle cells will be directed at: a) elucidating the mechanism by which phosphorylation con-trols actin-mysoin interaction, b) exploring a possible role for this system in controlling cell division and/or cell proliferation.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01602-02 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

ENHANCED SURVIVAL DURING ACUTE MYOCARDIAL INFARCTION IN RESERPINE-TREATED DOGS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.M. Kent	Hd., Sec. Exptl. Physiology	CB NHLI
OTHER:	R.A. Goldstein	Student	CB NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

SUMMARY OF WORK (200 words or less - underline keywords)

Reserpine administration, in doses equivalent to .25 mg b.i.d. po in man, have been shown to significantly reduce the incidence of spontaneous ventricular fibrillation after coronary occlusion during experimental myocardial infarction. These studies may provide the basis for a therapeutic trial in humans with coronary artery disease who are at high risk of sudden death.

591

PHS-6040
(12-75)

We previously demonstrated that both increased cholinergic and decreased adrenergic neural tone decreases the incidence of spontaneous ventricular fibrillation that occurs during acute coronary occlusion in dogs. Thus, vagal stimulation decreased the incidence of spontaneous ventricular fibrillation after occlusion of the left anterior descending and septal coronary arteries from 85% in control animals to 53% ($p<.01$) in animals with vagal stimulation. Conversely, pretreating animals with 6-OH-dopamine, which decreases adrenergic tone by depleting cardiac norepinephrine stores, decreased the incidence of spontaneous ventricular fibrillation from 80% in control animals to 20% in treated animals ($p<.01$). To determine the efficacy of an antiadrenergic agent that has the potential of being given orally to patients with coronary disease, we studied the effects of reserpine. There is considerable clinical experience with oral doses of reserpine of 3.5 to 7 µg/kg body weight/day. We therefore chose this dose range to be administered to experimental animals for 10-14 days prior to coronary artery occlusion.

Male mongrel dogs weighing between 20 and 34 kg were randomly assigned to one of three treatment groups: control (saline), low dose reserpine (3.5 µg/kg/ day) and high dose reserpine (7 µg/kg/day). At the time of study, the animals were anesthetized with sodium pentobarbitol (30 mg/kg) and intubated. Baseline heart rate, arterial and left atrial pressures were determined. The hearts were paced at 180 beats/min. The left anterior descending and septal coronary arteries were then occluded and the animals were observed for 30 minutes or until ventricular fibrillation occurred. Biopsies of all four chambers of the heart were taken for norepinephrine determinations.

Prior to pacing, the heart rate of the control animals averaged 175 beats/min. Animals receiving low-dose reserpine averaged 170 beats/min, (NS) and animals receiving high-dose reserpine averaged 140 beats/min ($p<.05$). Mean arterial pressure after coronary occlusion fell 6% in the control animals and 18% in both groups of reserpinized animals ($p<.01$). Left atrial pressure increased in the control animals from an average of 5 mm Hg prior to occlusion to 12 mm Hg at 10 minutes after occlusion. The average increase in left atrial pressure in the reserpinized animals was less, from a control of 5 mm Hg to 8 mm Hg at 10 minutes after occlusion. However, the increase in left atrial pressure was not significantly different than the control animals. Neuronal norepinephrine concentration in the left ventricle averaged .97 µg/g in control animals and reserpine treatment decreased neuronal norepinephrine to an average of .08 µg/g. There was no significant difference in the norepinephrine concentration of the animals receiving low-dose reserpine than those receiving high-dose reserpine. The incidence of spontaneous ventricular fibrillation after coronary occlusion was 78% in the control animals, 78% in the animals receiving low-dose reserpine (NS) but decreased to 45% in the animals which had received the high-dose reserpine ($p<.05$).

These results demonstrate that catecholamine depletion achieved with doses of reserpine that are comparable to those given to patients, is protective against ventricular fibrillation that occurs during acute experimental myocardial infarction.

It is not clear from these studies why the lower dose of reserpine is not protective against spontaneous ventricular fibrillation. It could be that there was significantly more catecholamine depletion achieved with the higher dose but this difference was not measureable because of the sensitivity of the techniques at such low levels of norepinephrine. An alternative explanation is that the higher dose of reserpine caused a greater degree of peripheral norepinephrine depletion and consequently a lower level of circulating norepinephrine; the significance of such an effect is that circulating catecholamines also have been shown to reduce ventricular electrical stability. It would appear from these experimental studies that clinical trials might be warranted to evaluate the potential antiarrhythmic actions of reserpine in patients who are at high risk of sudden death from coronary artery disease.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01614-02 CB

PERIOD COVERED
July 1, 1975 to June 30, 1976.

TITLE OF PROJECT (80 characters or less)
Reduction of ischemia by nitroglycerin and methoxamine: mechanisms of action.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	H.J. Smith	Guest Worker	CB NHLI
OTHER:	R.A.Goldstein	Medical Student	CB NHLI
	K.M. Kent	Hd., Sec. Exptl. Physiology	CB NHLI
	R. Aamodt	Hd., Sec. Whole Body Counter	Dept. Nuclear Med.
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)
Dept. of Nuclear Medicine

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	.55	.05

SUMMARY OF WORK (200 words or less - underline keywords)

Nitroglycerin reduces ischemic injury when administered following coronary artery occlusion, by increasing collateral blood flow and reducing the oxygen demand of the heart. This salutary effect may be offset by nitroglycerin-induced tachycardia, in which case maintenance of blood pressure and lowering of heart rate by methoxamine potentiates the effects of nitroglycerin.

PHS-6040
(12-75)

Project Description: To determine the mechanisms of the known beneficial action of nitroglycerin (TNG) and methoxamine (M) in acute myocardial infarction (AMI), myocardial blood flow (MBF) and ST segment elevation (ST) were measured after acute coronary artery occlusion in closed chest sedated dogs. MBF was measured with radioactive microspheres and ST was recorded from chronically implanted intramyocardial left ventricular electrodes. MBF at the center of the infarct was used as an index of collateral blood flow (CBF). During AMI, CBF averaged 18% of flow to nonischemic myocardium, but increased to 30% in 12 of 16 dogs given TNG. Enhanced CBF, with one exception, was associated with a decrease in ST. CBF diminished or remained unchanged in 4 dogs given TNG: ST increased in 3 in which tachycardia occurred. M was given to 9 dogs 20 minutes after TNG with no consistent change in CBF. ST increased slightly in 3 dogs which had marked TNG-induced improvement, but decreased in 6 dogs which had little or no TNG-induced change. Improvement followed slowing of heart rate and was associated with increased CBF or occurred when flow to nonischemic myocardium diminished, possibly reflecting a decrease in MVO_2. A significant multiple regression was found which described the determinants of ST change (ST):

$$\%\Delta ST = .41\ (\pm.19)\Delta HR - .21\ (\pm.06)\ \%\Delta CBF - 13.9$$

where %ΔCBF is percentage change in CBF: ΔHR = change in heart rate. Thus, TNG administered during AMI increases CBF and decreases ST except where excessive tachycardia or hypotension occur. In these cases, improvement occurs when M is added.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01630-02

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effects of Nitroprusside and Nitroglycerin on Ischemia After Coronary Occlusion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.S. Pearlman	Clinical Associate	CB NHLI
	R.L. Engler	Clinical Associate	CB NHLI
OTHER:	K.M. Kent	Hd., Sec. Exptl. Physiology	CB NHLI
	R.A. Goldstein	Medical Student	CB NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.08	.02

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of two commonly used coronary vasodilators on the degree of myocardial ischemia were studied. These two agents produced opposite effects on ischemic injury even though the hemodynamic effects of the agents were the same: Nitroglycerin decreased myocardial ischemia while nitroprusside increased ischemia.

576

PHS-6040
(12-75)

Project Description: Recent studies have generated considerable enthusiasm for the use of the nitroglycerin (TNG) and nitroprusside (NP) in the treatment of patients with acute myocardial infarction. These agents appear to improve hemodynamic function by their peripheral dilator effects on systemic arteries and veins, which reduce arterial and left ventricular filling pressures and thereby diminish left ventricular work. In addition, TNG reduces resistance to coronary collateral flow and increases coronary flow to ischemic areas. Thus, TNG appears to have two effects that would contribute to an amelioration of ischemia. However, no information is available relating to the direct effects of nitroprusside on coronary arterial vessels. We therefore examined the ability of nitroprusside to modify the extent of experimentally induced acute myocardial ischemia, and compared the effects of this drug with those of nitroglycerin.

Eight dogs with pre-existing multiple coronary occlusions underwent acute balloon occlusion of the left anterior descending coronary artery. The extent of ischemic injury was determined by summating ST segment elevation (ΣST) from 7 intramyocardial electrodes previously placed in the ischemic zone; measurements were made of heart rate, cardiac output, arterial pressure and left atrial pressure. Each animal was subjected to three occlusions in random order: 1) control (untreated), 2) following treatment with nitroprusside, 3) following treatment with nitroglycerin. Under control conditions, heart rate, arterial pressure, and left atrial pressure remained stable over the course of the 15 minute occlusion. ΣST rose from preocclusion levels to a peak value at 10 minutes, and remained stable over the next five minutes of occlusion. Nitroprusside caused an average reduction in mean arterial pressure of 25% and an increase in heart rate of 20%. These changes were accompanied by an increase in ΣST over control averaging 24% at 10 minutes and 33% at 15 minutes of occlusion ($p<0.05$). Nitroglycerin reduced arterial pressure 17% from control values and increased heart rate 20%. Despite causing nearly identical hemodynamic effects as nitroprusside, however, nitroglycerin administration was accompanied by an average reduction in ΣST below control values of 17% at 10 minutes ($p<0.05$) and of 11% at 15 minutes. In a subgroup of four dogs which had identical falls in arterial pressure and increases in heart rate following NP and TNG, the results were similar. Thus in dogs with pre-existing multiple coronary occlusions, none of whom had left ventricular failure, the extent of ischemic injury following experimental acute coronary occlusion is further aggravated by administration of nitroprusside, in contrast to the beneficial effect exerted by nitroglycerin. While perhaps not directly applicable to the clinical situation, these results suggest that the reduction in left ventricular work consequent to nitroprusside administration may not be accompanied by a reduction in ischemic injury; this finding emphasizes the point that therapy for acute myocardial infarction should be assessed in relation to its effects not only on hemoydnamic function, but also on the magnitude of ischemic injury.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01632-02

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effects of Vasodilators on Coronary Collateral Flow

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

N.L. Capurro	Pharm.Research Assoc.Trainee	CB	NHLI
K.M. Kent	Hd., Exptl. Sec. Physiololgy	CB	NHLI
S.E. Epstein	Chief, Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.3	.2

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of nitroglycerin, nitroprusside and phentolamine on coronary collateral function were tested in dogs with well-developed coronary collateral vessels. Nitroglycerin enhanced collateral flow by both a direct action on the coronary circulation and by indirect mechanisms. Although nitroprusside was more potent than nitroglycerin in reducing systemic arterial blood pressure, it was less effective in increasing coronary collateral flow. Phentolamine failed to increase collateral flow.

598

PHS-6040
(12-75)

Project Description: Studies in dogs and man indicate that nitroglycerin enhances collateral flow, an effect that has been shown to reduce ischemic injury during acute myocardial infarction. In the present investigation, the effects of intravenous and intracoronary nitroglycerin were compared in order to determine whether nitroglycerin augments collateral function by a direct action on the coronary circulation or only by indirect mechanisms such as change in heart size. The influence of heart rate on the effect of nitroglycerin also was tested. Further, the effects of nitroglycerin on collateral function were compared with those of two other vasodilators which are currently used in the treatment of acute myocardial infarction, nitroprusside and phentolamine. Dogs of either sex received general anesthesia, the heart was exposed through a left thoracotomy, and an ameroid constrictor was placed around the anterior descending (LAD) branch of the left coronary artery. Two-4 weeks were allowed for development of collateral vessels. At the time of study, the dogs were anesthetized with sodium pentobarbital and the chest opened. A polyethylene cannula was inserted in the LAD distal to the ameroid. A Gregg cannula was inserted through the subclavian artery and secured in the left main coronary artery. Both the left main and the distal LAD were perfused with blood shunted from the dog's carotid artery. When inflow to the LAD cannula was clamped, peripheral coronary pressure (PCP) was measured and retrograde flow (RF) was collected from side-arms of the LAD cannula. Systemic arterial pressure, left atrial pressure, ECG, and coronary flow were monitored continuously. Drugs were administered by constant intracoronary infusion (through the Gregg cannula) or by constant intravenous infusion. Since RF and PCP vary directly with perfusion pressure, arterial pressure was held constant at approximately 100 mm Hg, by inflation or deflation of a silastic cuff placed around the descending aorta. In 24 dogs with ameroid constrictors, control RF ranged from 11 - 100 ml/min (mean 35) and control PCP ranged from 48 - 94 mm Hg (mean 69). [In 7 normal dogs control RF ranged from 1.1 - 6.2 ml/min (mean 2.8) and control PCP ranged from 19 - 38 mm Hg (mean 24.6).] Intracoronary nitroglycerin, 0.3 - 100 µg/min, produced dose-dependent increases in RF but no change in PCP. Intravenous nitroglycerin, 3 - 300 µg/min, produced dose-dependent increases in both RF and PCP. Thus, nitroglycerin augments retrograde flow by a direct effect on the coronary vasculature since this effect is observed when the drug is administered directly into the coronary arterial system. However, there is an additional improvement in coronary collateral function as demonstrated by the increase in PCP seen only when the drug is administered intravenously. This further augmentation of collateral function must be due to peripheral effects of nitroglycerin such as venous pooling and reduction of ventricular volume. Decreasing heart rate, by vagal stimulation, from 180 to 60 beats/min increased both RF and PCP, but the effect of nitroglycerin on these parameters was not influenced by heart rate. Nitroprusside, when administered i.c. in doses of 3 - 100 µg/min, produced dose-dependent increases in RF which were similar to those seen with i.c. nitroglycerin. As with i.c. nitroglycerin, i.c. nitroprusside did not change PCP. However, intravenous nitroprusside, 1-30 µg/min, failed to change RF significantly; increments in RF were produced only by higher doses (100 - 300 µg/min). The change in RF was significantly greater ($P<.05$) with nitroglycerin than with nitroprusside at the lower doses

(10-30 µg/min). Intravenous nitroprusside produced increases in PCP similar to those seen with nitroglycerin. Nitroprusside was considerably more potent than nitroglycerin, however, in decreasing systemic arterial pressure and in reducing total coronary resistance. Thus, although nitroglycerin and nitroprusside produce similar effects on collateral function when administered directly into the coronary system, nitroglycerin is a more potent agent in increasing collateral function when administered systemically. Furthermore, nitroprusside is a more potent dilator of both systemic and coronary arterioles. Thus, if nitroglycerin and nitroprusside were administered systemically to produce the same reduction in systemic arterial pressure, nitroprusside would produce less of an augmentation in collateral function and would lead to a greater possibility of coronary flow maldistribution from ischemic to non-ischemic beda.

In summary, 1) nitroglycerin enhances coronary collateral function by a direct coronary effect and this direct effect is further augmented by indirect mechanisms; 2) decreasing heart rate augments coronary collateral flow, but the effect of nitroglycerin on collateral flow is independent of heart rate; 3) although i.c. nitroglycerin and nitroprusside produce similar effects on collateral function, i.v. nitroglycerin is more effective than i.v. nitroprusside in augmenting collateral flow; 4) phentolamine may have deleterious effects on collateral function; and 5) the relative vasodilator potencies of nitroglycerin and nitroprusside vary in different vascular beds -- for a given reduction in systemic arterial pressure, nitroprusside is less effective in increasing RF.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01647-01 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Contractile Properties of Acutely Reperfused Myocardium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H.J. Smith	Guest Worker	CB NHLI
OTHER:	K.M. Kent	Hd., Sec. Exptl. Physiology	CB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	.45	.05

SUMMARY OF WORK (200 words or less - underline keywords)

Interruption of the blood supply to heart muscle for up to 10 minutes arrests contraction, but does not produce permanent damage. Recovery of heart function follows restoration of the blood supply (reperfusion) but late deterioration lasting for up to 24 hours then occurs. Drug treatment which reduces reperfusion damage may benefit patients recovering from heart surgery.

601

PHS-6040
(12-75)

Project Description: Left ventricular myocardium rapidly loses contractile function when rendered ischemic by coronary artery occlusion and permanent damage ensues when ischemia is sustained for more than 15 minutes. Other workers, however, have shown that reperfusion after 10 minutes of ischemia is associated with impaired contractile function for up to 24 hours. We measured regional contractility in ischemic and acutely reperfused myocardium in an attempt to delineate the patterns of contraction under these conditions.

Mongrel dogs were anesthetized with sodium pentobarbital and subjected to thoracotomy. A pair of ultrasonic crystals (diameter 2 mm) was implanted in the anterior left ventricular wall and the distance between (1.5-2.0 cm) was computed electronically.

Following coronary artery occlusion, hypokinesia rapidly develops and progresses to dyskinesia over 20-40 seconds. The hypokinetic pattern develops in mid-systole so that early after coronary occlusion the initial velocity of shortening is preserved. Systolic shortening still occurs, but it does so at the end of systole and in early diastole as the left ventricular pressure returns towards zero: late systolic shortening diminishes over 1 hour with sustained ischemia.

Reperfusion after five (6 dogs) and then 10 (6 dogs) minutes is associated with complete or nearly complete return of contractile function over 30-45 seconds, but then a late deterioration occurs. The pattern of contraction during "reperfusion damage" appeared to related, at least in part, to the duration of ischemia. Reperfusion damage also occurred in 2 dogs pretreated with 6-OH-dopamine (20 mg/kg) and in 2 dogs treated with propranolol (1 mg/kg), suggesting that the brief recovery of contractile function was not an artefact contingent upon catecholamine release.

After spontaneous or electrically stimulated ventricular premature beats, the pattern of reperfusion damage was not seen in the post-ectopic beat, but early systolic dyskinesia reappeared progressively in the next 5-6 beats. Neither bradycardia (induced by vagal stimulation) nor paired pacing altered the pattern of reperfusion damage, suggesting that the augmentation in contractile function of the post-ectopic beat in reperfused tissue is not due to an altered R-R interval or increased calcium ion flux.

In 4 dogs with reperfusion damage, contractile alternans was seen as severe dyskinesia alternating with mild or less severe dyskinesia. The alternating pattern of contraction was seen in the arterial pressure as pulsus alternans.

These findings are of clinical importance in patients recovering from cardiopulmonary bypass surgery who may have temporary abnormalities of cardiac function without permanent myocardial damage. The abnormal pattern of contraction in reperfusion damage appears to be distinct from that observed in damage due to pure ischemia, and may relate to alterations of cell membrane permeability. Studies to define whether interventions can reduce reperfusion damage will be based on these initial observations.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01648-01 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Selective Depression of Ischemic Myocardium by Verapamil

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H.J. Smith	Guest Worker	CB NHLI
OTHER:	R.A. Goldstein	Student	CB NHLI
	J.M. Griffith	Project Engineer	BEIB
	K. M. Kent	Hd., Sec. Exptl. Physiology	CB NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	.25	.05

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil in appropriate clinical doses, depresses contractility in ischemic myocardium while sparing function in normal tissue. In contrast, propranolol depresses both normal and ischemic muscle. Selective depression of ischemic myocardium by verapamil or another calcium antagonist may benefit patients with acute myocardial infarction since ischemic injury can be reduced by depressing contractility (and hence oxygen demand).

604

PHS-6040
(12-75)

Project Description: Verapamil has been shown to decrease myocardial ischemic injury. Its mechanism of action is unclear, however, since 1) reduction in ischemia was not associated with alterations in collateral flow and 2) verapamil depresses contractility of normal myocardial (and presumably MVO_2) only at high concentrations. Hence, to determine whether verapamil might reduce ischemic injury by selectively depressing ischemic myocardium, we examined the contractile function of normal and partially ischemic myocardium after administration of verapamil (0.02-0.2 mg/kg), and compared the changes following propranolol (0.01-1 mg/kg). Regional contractile function was examined in 10 open-chest dogs with pairs of ultrasonic crystals placed in normal and ischemic segments of left ventricle; ischemia was controlled by graded occlusion of a shunt connecting the carotid and left anterior descending coronary arteries. Controlled reduction in shunt perfusion pressure (40-55 mm Hg) resulted in mild hypokinesia. Verapamil depressed contractile function in the ischemic segment in 5/5 dogs but did not alter maximum velocity (max V) or end-diastolic length of the normal segment. However, whenever propranolol was given in doses sufficient to depress ischemic myocardium, contractile function of normal myocardium also decreased. We conclude that verapamil selectively depresses ischemic myocardium, whereas propranolol depresses both normal and ischemic tissue. Since ischemic injury can be decreased by reducing contractile function (and thereby MVO_2), these results may have important clinical implications in the management of patients with acute myocardial infarction.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01649-01 CB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The determinants of collateral blood flow and ST segment elevation following coronary artery occlusion in the dog.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	H.J. Smith	Guest Worker	CB NHLI
OTHER:	R.A. Goldstein	Medical Student	CB NHLI
	K.M. Kent	Hd,Sec.Exp.Phys.& Pharm.	CB NHLI
	S.E. Epstein	Chief,Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

Dept. of Nuclear Medicine-NIH

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	.35	.05

SUMMARY OF WORK (200 words or less - underline keywords)

Following coronary artery occlusion, the changes in myocardial blood flow, heart rate and blood pressure were correlated with ST segment elevation in the electrocardiogram (an index of tissue injury). Regression equations were developed which showed that 1) ischemic injury is determined by the balance of blood flow (oxygen supply) and work load (oxygen demand), 2) changes in these functions during ischemia can modify ischemic injury, and 3) collateral blood flow is influenced positively by arterial pressure and negatively by heart rate.

PHS-6040
(12-75)

Project Description:
Considerable data exist indicating that measurement of ST segment elevation (ST) provides a reliable index of the severity of myocardial ischemia. Recent studies, however, have shown that there is only a poor correlation between collateral blood flow (CBF) and epicardial ST in open-chest dogs. To determine the precise relation between CBF and ST under more physiologic conditions (closed chest sedated dogs) and employing intramyocardial rather than epicardial electrodes, we measured CBF with radioactive microspheres and recorded ST in 26 dogs. Although ST always was found in areas of myocardium with very low flow ($p<.001$) the correlation coefficient between CBF (ml/min/g) and ST (mv) was relatively weak ($r=.59$). However, a significant multiple regression analysis (MRA) of ST against CBF, heart rate (HR) and normal zone blood flow (nMBF, which was used as an estimate of MVO_2), gave a much better correlation: $ST = 12.66\ (\pm.97)\ CBF + 3.69\ (\pm.74)\ nMBF + 0.04 \pm 0.02\ HR + 5.27$ ($R=0.9$; $p<0.001$). In 5 dogs HR was slowed by vagal stimulation and later increased by atrial pacing. In these and 7 other dogs with spontaneous changes in HR, MRA showed a significant relation between changes in ST and changes in CBF and nMBF: $\%\Delta ST=0.24\ (+.10)\ \%\Delta nMBF - 0.19\ (\pm 0.06)\%\Delta CBF - 15.4$ ($R=.84$; $p<0.001$). MRA also suggested that changes in arterial pressure (ΔAP, mm Hg) and HR were determinants of ΔCBF: $\%\Delta CBF=2.04\ (\pm.93)\ \Delta AP - 0.58\ (\pm.28)\Delta HR + 21.5$ ($R=.66$, $p<.01$). These results indicate that 1) CBF is only one of several determinants of ischemic injury, 2) absolute ST and ΔST can be predicted by MRA of such determinants of myocardial O_2 perfusion and demand as CBF, MVO_2 and HR, and 3) CBF is influenced positively by AP and negatively by HR.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01650-01 CB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mannitol on Acute Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	: H.J. Smith	Guest Worker	CB	NHLI
OTHER	: R.A. Goldstein	Medical Student	CB	NHLI
	M.J. Shmuklarsky	Medical Student	CB	NHLI
	K.M. Kent	Hd,Sec.on Exp. Phys.& Pharm.	CB	NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)
Dept. of Nuclear Medicine, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	.55	.05

SUMMARY OF WORK (200 words or less - underline keywords)

Although mannitol has been shown to reduce myocardial infarct size when administered before or soon after coronary artery occlusion, we found that it had no effect when administered three hours after the onset of ischemia. In contrast, nitroglycerin/methoxamine increased collateral blood flow and reduced ST segment elevation (an index of tissue damage). Thus, mannitol, in contrast to nitroglycerin/methoxamine is unlikely to be effective in reducing infarct size in patients with acute myocardial infarction.

PHS-6040
(12-75)

Project Description: Hypertonic mannitol has been shown to reduce ST segment elevation (ΣST) and increase coronary collateral blood flow (CBF) when administered before or soon after brief periods of coronary occlusion. We studied closed-chest sedated dogs to determine whether later administration of hypertonic mannitol also reduces ΣST (recorded from myocardial electrodes) and increases CBF (radioactive microspheres) and whether hypertonic mannitol potentiates the known salutary effects of nitroglycerin/methoxamine. In 5 dogs sedated with diazepam and morphine, 100 ml of hypertonic mannitol (25%) was infused over 30 min beginning 3 hours after LAD coronary occlusion. Although serum osmolality increased 17 mosm, no changes occurred in either mean CBF or ΣST. In contrast, when nitroglycerin/methoxamine was given 3 hours post occlusion, in another group of 5 dogs, CBF increased from 0.17 + .03 to 0.23 +.04 ml/min/g ($p<.05$) and ΣST fell from 39+5 to 25+2 mv ($p<.05$). Administration of hypertonic mannitol at 3 1/2 hrs to the dogs given nitroglycerin/methoxamine did not change further either CBF or ΣST. We conclude that in contrast to the increase in CBF and reduction in ΣST produced by nitroglycerin/methoxamine when administered as late as 3 hours after coronary occlusion, no such salutary effects follow administration of hypertonic mannitol. Thus, these results cast doubt the concept that hypertonic mannitol might be generally effective in reducing infarct size in patients with acute myocardial infarction who present several hours after onset of symptoms.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01651-01 CB

PERIOD COVERED
July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regional Contractility: Effects of Pharmacologic Agents on Reperfusion Damage After Brief Periods of Coronary Artery Occlusion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H.J. Smith	Guest Worker	CB NHLI
OTHER:	K.M. Kent	Hd., Sec. Exptl. Physiology	CB NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	.35	.05

SUMMARY OF WORK (200 words or less - underline keywords)

Impairment of contractile function of the heart follows both interruption (ischemia) and restoration (reperfusion) of the blood supply to heart muscle. Reperfusion damage is not modified by agents which reduce ischemic damage but appears to follow changes in the energy-transfer systems within heart muscle cells. Manipulation of the substrate (fuel) of the heart may ameliorate the deleterious effects of reperfusion damage.

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PHS-6040
(12-75)

Project Description: We previously described the patterns of regional contraction when left ventricular myocardium was reperfused after brief periods of coronary occlusion. Shortly after release of a 5-10 minute occlusion there is almost complete recovery of function; however, deterioration, characterized by early systolic dyskinesia, appears progressively and becomes stable by 15 minutes. We have termed this pattern of deteriorating function "reperfusion damage" and postualted that it may be due to a process distinct from the biochemical aberrations caused by pure ischemia. In the present study, we examined the pattern of contraction following reperfusion in anesthetized mongrel dogs subjected to 10 minutes of coronary artery occlusion. Contractile pattern was determined by signals transmitted and received by a pair of ultrasonic crystals implanted into the left ventricle. Administration of a variety of pharmacologic agents allowed preliminary deductions concerning the causation and possible modification of reperfusion damage.

Pre-treatment with nitroglycerin/methoxamine or propranolol, both of which are known to reduce ischemia, did not alter reperfusion damage. These negative results suggest that reperfusion damage occurs when a given threshold of ischemic injury is attained, and that reduction of ischemia does not modify subsequent reperfusion injury, as long as the degree of ischemia still exceeds this critical threshold.

Increase of serum osmolality (by 17-18 mosmol) with hypertonic mannital did not reduce reperfusion damage, suggesting that the process responsible for disordered contraction is not a disorder of cell volume, as occurs following release after 40 minutes of ischemia.

The effect of intracoronary isoproterenol (0.1 μg) and a calcium ionophore (Lilly A23187; 2 mg) were examined. Both agents induced a transitory positive inotropic response, but deterioration of function followed the ionophore, whereas function following isoproterenol returned to baseline. This observation supports biochemical studies which have suggested that reperfusion-induced increase in calcium flux and permeability may interfere with mitochondrial function.

Therefore, three membrane-active agents were studied. Hydrocortisone - considered to stabilize the cell membrane - did not alter reperfusion damage when administered prior to a 10 minute period of coronary occlusion. Similarly, verapamil, which interferes with slow calcium flux, had no effect on reperfusion damage, suggesting that if increased calcium flux was contributing to the damage, the slow calcium-flux was not involved. Ruthenium red, a calcium ionophore blocker, did not prevent reperfusion damage, but induced marked contractile alternans. Finally, we examined the effects of glucose (25 grams administered over 30 minutes) and found a reduction of reperfusion damage.

These results support the thesis that reperfusion damage is due, at least in part, to an impairment of mitochondrial energy transformation induced by altered calcium permeability and excessive accumulation of intracellular calcium. Administration of agents which reduce ischemia or act on the cell membrane, do not appear to influence reperfusion damage, but substrate manipulation may ameliorate the deleterious effects of reperfusion on contractile function.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01654-01 CB

PERIOD COVERED
July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Nitroglycerin and Early Changes in Collateral Function Following Acute Coronary Occlusion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N.L. Capurro	Pharm.Research Assoc.Trainee	CB NHLI
OTHER:	K.M. Kent	Hd., Exptl. Sec. Physiology	CB NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI
	R. Aamodt	Chief,Whole Body Counter Sec.	N.M. CC

COOPERATING UNITS (if any)

Dept. of Nuclear Med., Clinical Center, NIH.

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.3	.1

SUMMARY OF WORK (200 words or less - underline keywords)

Coronary collateral flow was monitored over 5 hours following occlusion in untreated dogs, and in dogs treated for one hour (beginning 10 minutes post-occlusion) with nitroglycerin and methoxamine. In untreated dogs, collateral flow was unchanged over 5 hours following occlusion. Treatment with nitroglycerin and methoxamine significantly increased collateral flow, and the increase in flow was maintained over the 5 hours studied, despite the fact that treatment was discontinued at 70 minutes post-occlusion.

PHS-6040
(12-75)

Project Description: We have shown that nitroglycerin is capable of increasing collateral flow to ischemic myocardium following acute coronary occlusion, an action which is accompanied by a decrease in ischemic injury and a reduction in the incidence of ventricular fibrillation. It is not known, however, whether collateral flow returns to pre-treatment levels during continued coronary occlusion when the drug is discontinued, or whether the acute increase in collateral flow induced by nitroglycerin promotes an augmentation of collateral function that is sustained even after the drug is discontinued. The purpose of this study was to determine 1) the collateral flow patterns in untreated animals during the first five hours following coronary occlusion, 2) whether a 1-hour period of treatment with nitrogylcerin and methoxamine might permanently alter the course of development of collateral function. Regional coronary flows were monitored over 5 hours following acute coronary occlusion in two groups of dogs: 1) control animals (n=6) and 2) animals which received nitroglycerin and methoxamine for one hour but then received no further treatment (n=6). One week prior to study an inflatable balloon cuff was placed around the left anterior descending (LAD) coronary artery, just below the first major diagonal branch; an intramyocardial electrode was implanted in the area of potential ischemia; a pacing wire was attached to the left atrium; and a catheter was inserted in the left atrium. At the time of study, the dogs were sedated with morphine and diazepam, and heart rate was maintained between 80 and 100. After the LAD was occluded by inflation of the balloon cuff, radioactive microspheres were injected through the left atrial catheter 10 minutes, 70 minutes, 2 hours 30 minutes, and 5 hours post-occlusion. In the treated dogs, infusion of 200-400 μg/min nitroglycerin, i.v., was begun after the injection of the isotope at 10 minutes. Methoxamine, 0.5 mg i.v., was administered as needed to keep blood pressure and heart rate at pre-nitroglycerin levels. Treatment was discontinued after the isotope injection at 70 minutes. Epicardial (EPI), endocardial (ENDO), and transmural (TM) flows and ENDO/EPI flow ratios were determined in normal and ischemic zone myocardium for both groups. Ten minute post-occlusion (pre-treatment) TM flow in the untreated dogs averaged 1.15±.24 ml/min/g in the normal zone and .11±.05 ml/min/g in the ischemic zone; in the treated dogs it averaged .88±.10 ml/min/g in the normal zone and .14±.02 ml/min/g in the ischemic zone. In the untreated group ENDO/EPI ratio averaged 1.28±.06 in the normal zone and .31±.05 in the ischemic zone. In the treated group it averaged 1.37±.07 in the normal zone and .29±.03 in the ischemic zone. In untreated dogs, total collateral flow did not change over 5 hours following occlusion. EPI flow was constant, ENDO flow tended to decrease (but not significantly) and the ENDO/EPI flow ratio decreased further at 5 hours to .21±.05 ($p<.05$). In contrast, in dogs treated with nitroglycerin-methoxamine, collateral flow increased significantly ($p<.05$). TM flow in the ischemic zone of the treated group increased from .14±.02 to .27±.04 ml/min/g at 70 minutes post-occlusion. Both EPI and ENDO flows were higher, and the ENDO/EPI flow ratio increased to .53±.10 ($p<.05$). The increase in

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collateral flow was maintained over the 5 hours studied (TM at 5 hours = .26±.03 ml/min/g), despite the fact that treatment was discontinued at 70 minutes.

Hence, our results demonstrate that collateral flow is unchanged over 5 hours following acute coronary occlusion in untreated dogs. Treatment with nitroglycerin-methoxamine improves collateral flow, and the increase in collateral flow is maintained at least 4 hours after treatment is discontinued. These results suggest that short-term treatment with nitroglycerin and methoxamine may have a lasting effect on the development of collateral function and may, as a consequence, result in a decrease in myocardial injury following acute coronary occlusion.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01655-01 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effects of Vasoconstrictors on Coronary Collateral Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N.L. Capurro	Pharm. Research Assoc.Trainee	CB	NHLI
OTHER:	K.M. Kent	Hd., Sec. Exptl. Physiology	CB	NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of methoxamine and angiotensin on coronary collateral function were tested in dogs with well-developed coronary collateral vessels. Preliminary studies suggest that angiotensin reduces collateral flow. Further studies are necessary to determine whether methoxamine also reduces collateral flow.

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PHS-6040
(12-75)

Project Description: Responsiveness of the coronary collateral circulation to vasoconstrictor agents was tested by studying the effects of methoxamine and angiotensin on retrograde flow (RF) and peripheral coronary pressure (PCP) in dogs with well-developed coronary collateral vessels. Methoxamine vasoconstrictor effects are mediated by alpha adrenergic receptors; angiotensin produces vasoconstriction via non-adrenergic mechanisms. Dogs were studied 2-4 weeks after an ameroid constrictor was placed around the left anterior descending (LAD) coronary artery. At the time of study, the animals were anesthetized with pentobarbital. RF and PCP were measured from side arms of a cannula inserted in the LAD distal to the ameroid. Methoxamine, 1-300 µg/min, or angiotensin, 0.01-10 µg/min were administered through a cannula in the left main coronary artery (i.c.). Since RF and PCP are directly related to coronary perfusion pressure, systemic arterial pressure was held constant at approximately 100 mm Hg by inflating a silastic cuff placed around the descending aorta. Although in 7 dogs RF tended to decrease with methoxamine, the change was not significantly different from control values. PCP was unaltered by methoxamine. Angiotension decreased RF in a dose-dependent fashion in 5 dogs. At 10 µg/min RF decreased by approximately 20% ($p<0.05$, compared to control). Angiotensin in these doses produced no change in PCP. Methoxamine, 1-100 µg/min, did not alter total coronary flow; however, coronary flow was significantly ($p<.05$) reduced when methoxamine was infused at 300 µg/min, i.c. Angiotensin did not produce significant changes in total coronary flow. Thus, under conditions of constant perfusion pressure, angiotensin reduced RF, suggesting a decrease in coronary collateral function. Further studies are necessary to determine whether methoxamine also reduces coronary collateral function and therefore whether alpha adrenergic receptors are present in the collateral circulation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01657-01 CB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Propranolol-induced changes in regional coronary flow during acute myocardial infarction.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.S. Borer	Senior Investigator	CB	NHLI
OTHER:	N. Capurro	Pharm.Research Assoc.Trainee	CB	NHLI
	K.M. Kent	Hd.Sec.Exp.Physiol. & Pharm.	CB	NHLI
	S.E. Epstein	Chief,Cardiology Branch	CB	NHLI
	F. Barcia	Visiting Scientist	CB	NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

SUMMARY OF WORK (200 words or less - underline keywords)

Propranolol, a drug which has been shown to reduce injury to the heart during acute myocardial infarction (heart attack) previously has been shown to act by reducing the work of the heart, and hence its need for oxygen. The present study demonstrates that the drug also acts by increasing blood flow to the injured area, thus increasing the supply of much needed oxygen to the damaged muscle.

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PHS-6040
(12-75)

Project Description: Propranolol, a beta adrenergic blocking agent, reduces ischemic injury during acute myocardial infarction both in the experimental animal and in man. The drug is known to act at least in part by reducing heart rate and myocardial contractility, and thereby reducing myocardial oxygen demand. Other investigators, studying acute myocardial infarction in anesthetized open-chest dog models with very high and unphysiologic heart rates, have found that while propranolol causes a net reduction in ischemic injury, it does not change, or even diminishes blood flow to ischemic areas.

In the present study, a closed-chest, sedated dog model is used, with hemodynamic parameters within the usual physiologic range. Ten dogs have been studied. After 10 minutes of coronary occlusion, regional coronary flow is assessed utilizing radioactive microspheres. Propranolol is then administered intravenously in doses sufficient to slow heart rate approximately 25% without reduction in mean arterial pressure. After 10 minutes of propranolol therapy, regional coronary flow again is measured. Over the succeeding 10 minutes, half the dogs are observed without further intervention and half are electrically paced to the heart rate which prevailed prior to propranolol administration. Regional coronary flow again is measured in all dogs.

As noted by others, we found that propranolol therapy uniformly reduces ischemic injury, as assessed by ST segment changes recorded by intramyocardial electrodes. However, in contrast to previous reports, collateral flow to ischemic areas consistently increases, often markedly, after propranolol administration. Concomitantly, blood flow to non-ischemic areas is reduced. Obviating propranolol's negative chronotropic effect with atrial pacing only partially reverses the drug's effect on collateral flow. Hence, other mechanisms, in addition to heart rate slowing, contribute to propranolol's ability to increase coronary flow. It is possible that a favorable redistribution of coronary flow occurs as a result of autoregulatory-induced coronary constriction, a change that probably occurs in the vascular bed supplying nonischemic myocardium, which experiences a reduction in MVO_2.

In summary, our results suggest that in addition to reducing myocardial oxygen demand, propranolol benefits the acutely ischemic myocardium by increasing oxygen supply.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01616-03 CB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of TNG during exercise in Valvular Heart Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	J. S. Borer	Senior Investigator	CB	NHLI
OTHER :	D.R. Redwood	Hd.Sec.Cardiovasc.Diag.	CB	NHLI
	R.E. Goldstein	Senior Investigator	CB	NHLI
	S.E. Epstein	Chief,Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.06	.05	.01

SUMMARY OF WORK (200 words or less - underline keywords)

Our results suggest that nitroglycerin therapy may be a useful adjunct in the pharmacologic management of patients with valvular heart disease by reducing exertional symptoms and increasing exercise tolerance.

620

PHS-6040
(12-75)

Project Description: Nitroglycerin reduces pulmonary arterial and left atrial pressures and pulmonary vascular resistance at rest in patients with mitral stenosis. We have observed similar changes in patients with aortic insufficiency and mitral insufficiency. Changes in cardiac output during such studies have been variable. To assess the potential clinical utility of nitroglycerin in patients with valvular heart disease we are determining the effect of nitroglycerin 1) on treadmill exercise capacity and 2) on the pulmonary artery pressure and cardiac output response to exercise. Thus far, 10 patients have been studied, most with mixed mitral and aortic valve lesions. Of these, 9 have had technically adequate evaluations of exercise tolerance; all have shown increases in exercise tolerance during nitroglycerin therapy as compared with placebo therapy; tolerance had increased an average of 25% after 0.5 mg sublingual nitroglycerin; five patients have undergone technically adequate evaluation of pulmonary artery pressure and cardiac output response to exercise. In all patients, pulmonary artery pressure has been lower and cardiac output higher at maximal exercise during nitroglycerin therapy than during placebo therapy.

Our results suggest that vasodilator therapy may be a useful adjunct in the pharmacologic management of patients with valvular heart disease by reducing exertional symptoms and increasing exercise tolerance.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01631-03 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Nitroglycerin Therapy for Acute Myocardial Infarction in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.S. Borer	Senior Investigator	CB NHLI
OTHER:	D.R. Redwood	Hd., Sec. on Cardiovasc. Diag.	CB NHLI
	B. Levitt	Prof. of Medicine -New York Med.College	
	S.E. Epstein	Chief, Cardiology Branch	
	N. Cagin	Asst.Prof of Medicine- New York Med.College	
	H. Vallin	Visiting Fellow in Cardiology-Guys Hosp.,London,England.	

COOPERATING UNITS (if any)

Cardiology Dept., Flower & Fifth Ave. Hospitals, New York, N.Y.
Guy's Hospital, London, England

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	0

SUMMARY OF WORK (200 words or less - underline keywords)

Nitroglycerin has been found to be useful in reducing heart muscle injury when given to patients early during acute myocardial infarction. In patients with normal cardiac mechanical function during heart attack, the addition of phenylephrine to prevent a fall in blood pressure seems to potentiate the beneficial effects of nitroglycerin, while in patients with subnormal cardiac mechanical function ("heart failure") nitroglycerin alone seems to be most effective.

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PHS-6040
(12-75)

Project Description: Previous investigations in dogs have demonstrated that nitroglycerin reduces ischemic injury and enhances ventricular electrical stability during acute coronary occlusion. These beneficial effects of nitroglycerin are potentiated by preventing drug-induced hypotension with an alpha-adrenergic agonist. Such combination therapy also has been found to reduce the incidence of spontaneous postocclusion ventricular fibrillation in dogs. The present study was designed to determine the efficacy of nitroglycerin, alone and with its hypotensive effects prevented with phenylephrine (an alpha agonist), in reducing the extent and severity of myocardial injury sustained during acute myocardial infarction in man. The efficacy of these interventions was assessed by ST segment analysis of 35-lead precordial surface maps (experimental studies have demonstrated that changes in ST segment elevation reflect changes in myocardial ischemic injury).

Twelve patients with acute myocardial infarction were studied. ST segment abnormalities were reduced in each (17 to 60%) during therapy with sublingual nitroglycerin and phenylephrine. However, the optimal therapeutic regimen depended upon whether or not the patient was in left ventricular failure.

In the seven patients without left ventricular failure, nitroglycerin-administered alone caused a fall in blood pressure and a reflexly mediated increase in heart rate. These changes were not associated with a consistent decrease in the degree of ST segment elevation. In one patient it caused an excessive tachycardia, which led to an increase in the degree of ischemic injury. In contrast, when phenylephrine was added and infused at a rate sufficient to reverse the blood pressure lowering effects of nitroglycerin, significant improvement in the degree of myocardial ischemia occurred in each patient.

In the five patients with left ventricular failure, nitroglycerin alone led to an average 15 mm fall in PA wedge pressure and a 26 mm Hg fall in mean arterial pressure. Despite the fall in arterial pressure, there was no reflex tachycardia. Moreover, in contrast to the patients without failure, TNG alone uniformly improved ST segment abnormalities. After abolition of the TNG induced arterial pressure fall with phenylephrine, significant ST segment improvement was still present, but the benefit tended to be less marked than with nitroglycerin alone.

We conclude that ischemic injury occurring during acute myocardial infarction often, but not always, is reduced by nitroglycerin alone. When nitroglycerin and phenylephrine are given, ischemia is usually if not always diminished, with minimal risk of adverse effects. However, response of ischemia to therapy is not homogeneous, and cannot be predicted with certainty by monitoring hemodynamic variables alone. Therefore, at present it appears that while general guidelines, based on the presence or absence of failure,

can be employed when initiating vasodilator therapy in the patient with an acute infarct, optimal therapy in the individual patient can be achieved only if a method such as precordial mapping is utilized for objective evaluation of ischemia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01661-01 CB

PERIOD COVERED

July 1, 1975, through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Natural History of Coronary Artery Disease in Mildly Symptomatic Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.M. Kent	Hd., Sec. Exptl. Physiology	CB NHLI
OTHER:	D.D. Savage	Clinical Associate	CB NHLI
	J.S. Borer	Senior Investigator	CB NHLI
	S.B. Itscoitz	Asst. Hd., Sec. Cardiovasc. Diagnosis	CB NHLI
	D.R. Redwood	Hd., Sec. Cardiovasc. Diagnosis	CB NHLI
	M.V. Green	Chief, Applied Physics Sec., Dept. Nuclear Medicine	
	J.J. Bailey	Hd., Med. Applications Sec., Div. Computer Res.	
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

Department of Nuclear Medicine

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.1	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with coronary artery disease who are only mildly symptomatic are being assessed to determine if a subgroup of patients who are at high risk of sudden death can be identified. More aggressive therapeutic trials would be justified in such a subgroup of individuals.

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PHS-6040
(12-75)

Project Description: The management of patients with coronary artery disease is currently being reevaluated since coronary artery revascularization procedures lead to such excellent symptomatic improvement. However, this operation has not been shown to increase longevity or prevent subsequent myocardial infarction. Thus, the role of this operation in the management of patients who have coronary artery disease but who are only mildly symptomatic is unknown. The only data available in the literature on the natural history of patients with coronary artery disease have been accumulated by centers which have active operative intervention programs. Thus, the patients who make up the "control groups" are highly selected individuals who usually have some complicating process which precludes operative intervention. The present investigation was set up as a prospective study of patients with coronary artery disease who are only mildly symptomatic to determine 1) whether low risk and high risk subgroups can be identified and 2) if a high risk subgroup can be identified, whether the annual mortality is sufficiently high enough to warrant consideration of a randomized study in which the results of medically treated and operatively treated groups are compared.

To date 35 patients have been admitted to this study. An additional 12 patients who were referred because of atypical chest pain were found to have normal coronary arteriograms and were thus not admitted to the study. The following investigations are being performed: 1) Cardiac catheterization, including selective left and right coronary arteriograms and left ventricular cineangiograms. Evaluation of the number of coronary vessels involved and extent of involvement is being determined. Left ventricular function is evaluated from the left ventricular cineangiogram. Vagal responses are assessed by examining the reflex decrease in heart rate in response to increased systolic arterial pressure produced by phenylephrine infusion.

2) Isotope angiography (radionuclide angiography). Ten mCi of technetium^{99} labelled human serum albumin is injected and a gamma scan of the heart is obtained. With this procedure, left ventricular function can be evaluated in a noninvasive manner. It is possible that this totally noninvasive technique will be important in identifying a high risk subgroup because of the development of sequential abnormalities of left ventricular function. In addition, quantitative index of left ventricular function, the ejection fraction, can be obtained with this noninvasive technique.

3) Double isotope studies. Although coronary angiography defines anatomic defects, no information concerning the adequacy of myocardial perfusion at rest or during stress can be obtained with conventional techniques. Since the precise pathophysiologic significance of the broad spectrum of coronary artery narrowing is not known, it is important to determine whether a more reliable prognostic index of subsequent coronary death can be developed based on studies of the adequacy of myocardial perfusion.

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Myocardial perfusion can be assessed by the use of radioisotope labelled human albumin microspheres. The perfusion studies are performed in the following manner. Technetium ^{99} labelled human albumin microspheres or iodine ^{131} labelled macroaggregated albumin are injected into the left and right main coronary artery with the patient at rest. Then right atrial pacing is initiated and the heart rate gradually increased until either angina or 85% of the maximum predicted heart rate occurs. At that point, the other radionuclide is injected selectively into both coronary arteries and pacing discontinued. Injection sequence of the radionuclides is randomized for each patients. Following the removal of the catheter, the patient is transferred to the Nuclear Medicine Laboratory where myocardial imaging is carried out to obtain rest and stress scintigrams.

4) Exercise studies. Some studies suggest that ECG alterations occurring during exercise stress tests (ST segment elevation or marked depression, premature ventricular beats) may predict which patients with coronary artery disease are at a high risk. Thus, patients admitted to this study are exercised in the upright position on a bicycle ergometer at increasing work loads until 85% of predicted maximum heart rate or angina pectoris occurs. ST segments are measured during and after exercise. The frequency and temporal characteristics of premature ventricular beats also are determined.

5) Ambulatory and sleep electrocardiographic monitoring. Twenty-four electrocardiographic monitoring is performed during the second or third day of admission to determine the presence or absence of premature ventricular contractions during daily activities. During the 24 hour period, sleep electrocardiograms are assessed to determine the extent of sleep bradycardia and sleep arrhythmias. It has been demonstrated previously that vagal mechanisms are deficient in patients after myocardial infarction. The degree of sleep bradycardia is used as an index of vagal tone and to determine if this will correlate with subsequent sudden death.

Of the 35 patients admitted to the study, the average followup is six months. At the present time, there has been one death and two patients who have become more symptomatic necessitating operation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01628-03 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Sudden Infant Death Syndrome: Potential Cardiac Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.J. Maron	Senior Investigator	CB NHLI
OTHER:	C.E. Clark	Senior Investigator	CB NHLI
	R.E. Goldstein	Senior Investigator	CB NHLI
	R.B. Fisher	Coroner, State of Maryland	
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

Coroner's Officer, State of Maryland, Baltimore, Md.

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

SUMMARY OF WORK (200 words or less - underline keywords)

A considerable proportion of close relatives of infants dying of SIDS demonstrated prolongation of the QT interval on electrocardiogram (a finding that has been associated with sudden death in infants, children and adults). This observation suggests that cardiac mechanisms may play a role in some sudden, unexpected infant deaths.

628

PHS-6040
(12-75)

Project Description: To investigate the potential role of cardiac mechanisms in the genesis of sudden infant death syndrome (SIDS), 42 sets of parents who had an infant with SIDS were studied by ECG and echocardiography. Prolonged QT interval was present in one member of 12 (29%) parental sets. Prolonged QT interval was also present in 10 (40%) of 25 siblings of infants with SIDS (in families in which a parent had prolonged QT interval), suggesting an autosomal dominant pattern of inheritance. One infant with "near-miss" SIDS showed a markedly prolonged QT interval. Asymmetric septal hypertrophy (ASH) was present in one member of only two (5%) other parental sets. Myocardium was analyzed histologically in another group of 45 infants with SIDS and 26 control infants. Small foci of disorganized cardiac muscle cells, that resembled those in ASH but were much less marked in severity, were present in the ventricular septum of 22% of infants with SIDS and 12% of controls. Although the significance of these cells is unknown, they may serve as a nidus for ventricular arrhythmias in some susceptible infants. These findings suggest that inheritable cardiac mechanisms, particularly those related to prolonged QT interval syndrome, may play a role in a considerable proportion of sudden, unexpected infant deaths.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01633-02 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Echocardiographic abnormalities following closure of an atrial septal defect.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. S. Pearlman	Clinical Associate	CB	NHLI
OTHER:	J.S. Borer	Senior Investigator	CB	NHLI
	W.L. Henry	Senior Investigator	CB	NHLI
	C.E. Clark	Clinical Associate	CB	NHLI
	S.B. Itscoitz	Asst.Hd. Cath Lab.	CB	NHLI
	D.R. Redwood	Hd.Sec.Cardiovasc.Diag.	CB	NHLI
	S.E. Epstein	Chief,Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.03	.02	.01

SUMMARY OF WORK (200 words or less - underline keywords)

Six months after complete closure of atrial septal defect, two-thirds of patients have residual abnormalities of ventricular septal motion and right ventricular size by echocardiography. However, these echocardiographic abnormalities do not appear to have major functional significance, since all patients studied had normal exercise tolerance by standardized testing after operation.

630

PHS-6040
(12-75)

Project Description: Postoperative echocardiograms (echo) often demonstrate persistent right ventricular (RV) dilatation and paradoxic ventricular septal motion following repair of an atrial septal defect (ASD). To determine the cause and functional significance of these observations, 21 pts with ASD underwent preoperative and postoperative catheterization and echo studies. Preoperatively, pulmonary:systemic flow ratio ranged from 1.6:1 to 6.4:1. All pts had RV dilatation; septal motion was paradoxic in 16, flat in 3 and normal in 2. Postoperatively, 20 of 21 pts had no shunt and 15 of 21 had normal RV systolic pressure ($\leq$30 mm Hg). Septal motion was paradoxic in 7, flat in 7 and normal in 7. Although RV size decreased in 20 of 21 pts, it remained abnormal in 15 of 21 pts. Postoperative septal motion became normal only when RV size returned to normal, which occurred in 6 of 12 pts <25 years old at operation, but in 0 of 9 pts >25 years old. Postoperatively treadmill exercise capacity and maximal oxygen consumption (VO_2) were determined in 8 consecutive pts; all results were normal, despite the presence of persistent paradoxic or flat septal motion in 4 of the 8, and persistent RV hypertension in 1. We conclude that persistent RV dilatation and septal paradox after ASD repair are common in the absence of residual shunt even when RV pressure is normal; the persistence of these abnormalities is more common in pts with RV volume overload present for many years prior to repair. Although our data indicate that persistent RV dilatation has no short-term functional significance, its long-term cardiac effects are unknown.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01639-04 CB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Echocardiographic Characteristics of Infiltrative Cardiomyopathy.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.S. Borer	Senior Investigator	CB	NHLI
OTHER:	W.L. Henry	Senior Investigator	CB	NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.02	.01	.01

SUMMARY OF WORK (200 words or less - underline keywords)

The echocardiogram has been used to delineate a group of abnormalities of cardiac structure and function which are present consistently in many patients with infiltrative diseases known to involve the heart. These abnormalities include thickening of the ventricular walls and loss of ventricular compliance with concomitant maintenance of normal pump function until very late stage of disease.

PHS-6040
(12-75)

Project Description: Echocardiography (ECHO) was used to evaluate the cardiac status of adults with systemic diseases (amyloidosis, 4 pts; hemosiderosis, 5 pts; mucopolysaccharidosis, 4 pts; idiopathic hypereosinophilia, 10 pts) known to be associated with infiltrative cardiomyopathy. Though 8 (35%) pts had no other clinical or non-invasive laboratory evidence of cardiac disease, all had ECHO abnormalities consistent with infiltrative disease. LV mass was elevated (>275 gm) in 23 (88%), (mean 385 gm, nl 211, $p<.01$); LV free wall and septum were symmetrically thickened (>11 mm) in 23 pts (mean 14.5 mm, nl 9.8, $p<.01$); LV internal dimension was abnormal in 7 pts. Mitral valve closure slope was reduced (<125 mm/sec) in 20 pts (mean 87 mm/sec, nl 147, $p<.01$), consistent with diminished LV compliance. However, systolic function was well maintained (ejection fraction mean 71%, nl 65%, n.s.). These ECHO findings were commonly associated with each disease studied. We conclude that in adults with diseases known to be associated with infiltrative cardiomyopathy 1) ECHO is a sensitive technique for detecting abnormalities in patients with clinically inapparent cardiac disease, 2) ECHO studies frequently demonstrate symmetrically increased LV wall thickness and LV mass and 3) systolic function is preserved while diastolic compliance is reduced.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01640-03 CB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Limitations of the ECG Response to Exercise in Predicting Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.S. Borer	Senior Investigator	CB NHLI
OTHER:	J.F. Brensike	Coordinator, NHLI Type II Coronary Intervention Study - MD	
	D.R. Redwood	Hd., Sec. Cardiovasc. Diagnosis	CB NHLI
	S.B. Itscoitz	Asst. Hd. Cath Lab.	CB NHLI
	E.R. Passamani	Staff Associate	MDB NHLI
	N.J. Stone	Staff Associate	MDB NHLI
	J.M. Richardson	Staff Associate	MDB NHLI
	R.I. Levy	Director, NHLI	NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

Molecular Disease Branch, NHLI
Division of Heart and Vascular Disease, NHLI

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.2	.05

SUMMARY OF WORK (200 words or less - underline keywords)

Electrocardiography during exercise traditionally has been used to help determine the presence or absence of coronary artery disease in a given individual. Our results demonstrate that the results of such testing are relatively insensitive in patients with symptoms of coronary disease, and are very poor predictors of coronary artery disease in subjects without symptoms.

PHS-6040
(12-75)

Project Description: The electrocardiographic (ECG) response to graded bicycle exercise (to 85% of predicted maximal heart rate) was evaluated prior to coronary angiography in 89 patients, aged 21 to 55 years, with type II hyperlipoproteinemia. Patients were studied if they had a) a history of myocardial infarction and/or typical angina (43 patients), b) "atypical angina" (16 patients), or c) a positive ECG response during or shortly after exercise, but were otherwise without evidence of cardiac disease (30 patients). Of the 43 Group A patients, 39 had $\geq$50% stenosis; however, 26 (67%) of these 39 had negative ECG tests, including 9 of 17 patients with >50% stenosis in each of 3 vessels. Of the 16 Group B patients, 5 had $\geq$50% stenosis, 3 with positive ECG tests; 1 patient had a positive test but normal arteriogram. Of the 30 Group C patients, 11 (37%) had $\geq$50% stenosis; however, 9 (30%) had minor stenoses ($\leq$50%) and 10 (33%) had entirely normal coronary arteries. Therefore, exercise electrocardiography results in a high frequency of both false negative responses (in patients with clinically suspected coronary disease) and false positive responses (in asymptomatic patients). We conclude that while exercise electrocardiography may be of value in epidemiologic studies, its applicability as a diagnostic tool in the individual patient has marked limitations.

Publications: New Engl J Med 293: 367-371, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01646-01 CB

PERIOD COVERED
July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)
CHARACTERISTICS OF DISPROPORTIONATE SEPTAL THICKENING IN PATIENTS WITH ASSOCIATED HEART DISEASE

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.J. Maron	Senior Investigator	CB NHLI
OTHER:	C.E. Clark	Senior Investigator	CB NHLI
	W.L. Henry	Senior Investigator	CB NHLI
	J.E. Edwards	Hd., Dept. Pathology, Miller Hospital, St. Paul, MI	
	R. Fukuda	Special Fellow, Dept. Path., Miller Hosp., St. Paul	
	E.C. Mathews	Special Fellow, Georgetown Univ., Washington, D.C.	
	D.R. Redwood	Hd., Sec. on Cardiovasc. Diag.	CB NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)
United Hospitals-Miller Division, Department of Pathology, St. Paul, Minn.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.6	

SUMMARY OF WORK (200 words or less - underline keywords)

Disproportionate thickening of the ventricular septum (a finding characteristic of hearts with genetically transmitted ASH) was present in about 10% of hearts from patients with a variety of congenital or acquired heart diseases. The absence of disorganized myocardial cells in the ventricular septum of these patients and the rare occurrence of disproportionate septal thickening in their close relatives suggests that the disproportionate septal thickening is probably secondary to their basic heart condition.

PHS-6040
(12-75)

Project Description: Asymmetric septal hypertrophy (ASH) is a genetically transmitted cardiac condition characterized by a disproportionately thickened ventricular septum that contains numerous disorganized cardiac muscle cells. To determine the prevalence of disproportionate septal thickening (DST) in adults and children with a variety of acquired or congenital cardiac diseases, observations were made on 278 patients with LV or RV overload, including 171 studied by echocardiography (ECHO) and 107 studied at necropsy. Overall, DST was present in 13% of the 278 patients. Specifically DST was present in 50% of patients with pulmonary stenosis, 26% of patients with primary pulmonary hypertension, 11% of patients with Eisenmenger syndrome or aortic valve disease and 7% of patients with mitral valve disease. DST was not present in patients with atrial or ventricular septal defects and left-to-right shunts. In patients with RV overload, prevalence of DST correlated with increasing RV systolic pressure. None of the patients with DST studied at necropsy showed disorganized cardiac muscle cells (characteristic of genetically transmitted ASH) in the ventricular septum. DST was demonstrated by ECHO in only 1 of 60 first degree relatives of patients with DST and other associated cardiac diseases. Thus, DST occurs in about 13% of patients with a variety of other cardiac diseases, but is most common in patients with severe RV overload. Absence of disorganized cardiac muscle cells in the ventricular septum of patients with DST and the rare occurrence of DST in relatives of these patients suggests that septal thickening occurring in patients with cardiac lesions producing RV or LV overload is not usually a manifestation of genetically transmitted ASH.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01652-01 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Discrepancies Between Echocardiographic and Necropsy Measurements of Ventricular Wall Thicknesses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.J. Maron	Senior Investigator	CB	NHLI
OTHER:	W.L. Henry	Senior Investigator	CB	NHLI
	W.C. Roberts	Chief, Section of Pathology		NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)

Section of Pathology

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Certain patients diagnosed as having disproportionate septal thickening by echocardiography during life appeared to have concentric thickening at necropsy examination. Such disparities are explained by the fact that the posterobasal left ventricular wall thickens considerably more than the ventricular septum in systole and postmortem hearts are often fixed in the systolic phase of the cardiac cycle.

638

PHS-6040
(12-75)

Project Description: Disproportionate thickening of the ventricular septum in relation to the posterobasal left ventricular wall is the characteristic echocardiographic marker of patients with asymmetric septal hypertrophy (ASH). However, in several patients with disproportionate septal thickening diagnosed by echocardiography (ventricular septal to posterobasal left ventricular wall thickness ratios of $\geq$1.3), we discovered marked discrepancies between echocardiographic and necropsy measurement of wall thicknesses that led to uncertainty regarding the actual cardiac diagnosis. To resolve these apparent incongruities, the echocardiograms of 18 patients with heart disease who subsequently died were studied, including nine with disproportionate septal thickening and nine with concentric thickening or normal wall dimensions; measurements of ventricular wall thicknesses and septal-free wall thickness ratios obtained by echocardiography and at necropsy were compared.

Six of seven patients with mildly abnormal septal-free wall ratios of 1.3 or 1.4 during life had septal-free wall ratios that were not diagnostic of disproportionate septal thickening at necropsy; one patients had a mildly abnormal septal-free wall ratio of 1.3 both during life and at necropsy. Two other patients had markedly increased septal-free wall ratios of $\geq$2.0 during life; the septal-free wall ratios measured at necropsy in these two patients were markedly reduced but still abnormal (i.e., $\geq$1.3).

Discrepancies between ventricular wall thicknesses and septal-free wall ratios obtained during life and at necropsy in patients with disproportionate septal thickening may be explained by the following facts: 1) echocardiographic measurements during life were made in diastole as per convention, but measurements at necropsy were made in hearts that appeared to have been fixed in the systolic phase of the cardiac cycle; 2) the posterobasal left ventricular wall thickens considerably more than the ventricular septum in systole, as determined by echocardiography. Specifically, posterobasal left ventricular wall thicknesses in these patients increased from diastole to systole (mean systolic thickening, 42.0$\pm$4.7%) to a much greater extent that did ventricular septal thickness (mean systolic thickening, 9.5$\pm$3.1%; $p<0.001$). This phenomenon resulted in septal-free wall ratios in systole (mean 1.2$\pm$0.05) that were consistently smaller than those in diastole (mean 1.5$\pm$0.11; $p<0.005$). Furthermore, septal-free wall ratios obtained at necropsy (mean 1.2$\pm$0.07) corresponded most closely to ratios obtained by echocardiography in systole.

In contrast, the nine patients without disproportionate septal thickening had septal-free wall ratios that did not differ significantly whether measurements were obtained echocardiographically in diastole (mean 1.0$\pm$0.03) or in systole (mean 1.0$\pm$0.02), or were obtained at necropsy (mean 1.0$\pm$0.02). In these patients, the increase in posterobasal left ventricular wall thickness from diastole to systole (mean percent change, 27.4$\pm$4.9%) did not differ significantly from the increase in ventricular septal thickness (mean percent change, 24.3$\pm$5.4%). Therefore, our data show that assessment of

ventricular wall thicknesses and septal-free wall ratios obtained by echocardiography in diastole may differ from those obtained at necropsy and that echocardiographic assessment in diastole provides a more sensitive method than necropsy examination for documenting the presence of disproportionate septal thickening.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01653-01 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Tunnel Subaortic Stenosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.J. Maron	Senior Investigator	CB	NHLI
OTHER:	D.R. Redwood	Hd., Sec. Cardiovascular Diagnosis	CB	NHLI
	W.C. Roberts	Chief, Section of Pathology		NHLI
	W.L. Henry	Senior Investigator	CB	NHLI
	A.G. Morrow	Chief, Clinic of Surgery		
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)

Section of Pathology

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Eleven patients with an unusual form of left ventricular outflow obstruction, in which the characteristic anatomic finding is diffuse tubular narrowing of the outflow tract, were evaluated. Such patients with tunnel subaortic stenosis should be distinguished from other patients with left ventricular outflow obstruction because of the ineffectiveness of operation in this condition.

641

PHS-6040
(12-75)

Project Description: The clinical and morphologic features of tunnel subaortic stenosis, an unusual form of obstruction to LV outflow, are described in nine patients. Patients ranged in age from 7 to 34 years (avg 18). The most characteristic anatomic feature of this congenital cardiovascular malformation is marked tubular narrowing of the LV outflow tract (1-3 cm in length) that remains fixed during the cardiac cycle. The aortic anulus was abnormally small in 5 of the 9 patients, including one patient who also had a hypoplastic ascending aorta and coarctation of the aorta. Evidence of a small mitral orifice was present in two patients. Two other patients also had evidence of genetically transmitted asymmetric septal hypertrophy. The preoperative LV outflow gradient ranged from 60 to 160 mm Hg (avg 125). Operation (resection of subvalvular muscle and/or fibrous tissue) in seven patients reduced the gradient minimally or not at all in 5 and greatly (over 100 mm Hg) in two; however, all patients had residual gradients postoperatively (range 50 to 200 mm Hg; avg 110). Two patients died at operation and three others died suddenly and unexpectedly; each of these three patients had minimal or no symptoms. Thus, the anatomic features of tunnel subaortic stenosis clearly distinguishes this condition from other forms of fixed LV outflow obstruction, including discrete (membranous) subaortic stenosis and valvular aortic stenosis. Because of the apparent ineffectiveness of current operative methods in patients with fixed diffuse LV outflow obstruction, it is essential to differentiate this condition from other forms of osbstuction to LV outflow.

Publications: None

642

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01656-01 CB

PERIOD COVERED

July 1, 1975, to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Disproportionate septal thickening in coronary artery disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.J. Maron	Senior Investigator	CB NHLI
OTHER:	D.D. Savage	Clinical Associate	CB NHLI
	C.E. Clark	Clinical Associate	CB NHLI
	W.L. Henry	Senior Investigator	CB NHLI
	J.E. Edwards	Hd., Dept. Pathology, Miller Hosp.,	St. Paul, MI
	D.R. Redwood	Hd., Sec. on Cardiovasc. Diag.	CB NHLI
	S.E. Epstein	Chief, Cardiology Branch	CB NHLI

COOPERATING UNITS (if any)

United Hospitals-Miller Division, Dept. of Pathology, St. Paul, Minn.

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Disproportionate thickening of the ventricular septum (a finding characteristic of hearts with genetically transmitted ASH) was present in about 10% of patients with coronary artery disease. The finding that disproportionate septal thickening could be detected in only some of these patients' families suggests that the septal thickening may either be genetically transmitted or "secondary" to the underlying cardiac disease.

643

PHS-6040
(12-75)

Project Description: Asymmetric septal hypertrophy (ASH) is a genetically transmitted cardiac condition characterized by a disproportionately thickened ventricular septum. Symptoms of this genetic disease are remarkably similar to those caused by coronary artery disease. Hence, when the two conditions coexist, it is difficult to dissect out which abnormality is playing the most important clinical role. However, disproportionate septal thickening also occurs as a result of other types of heart disease. When it does, it is a secondary phenomenon and not a primary cause of symptoms. The present study, therefore, was undertaken to determine 1) the prevalence of disproportionate septal thickening in patients with coronary artery disease and 2) whether the septal thickening is genetically transmitted.

Echocardiographic studies were performed on 88 patients with angiographically documented coronary artery disease. Disproportionate septal thickening was present in 9% of the 88 patients. It was also detected in 5 of 21 first degree relatives in these families studied by echocardiography; relatives with disproportionate septal thickening were present in three of the 8 families studied. Thus, these data show that disproportionate septal thickening occurs in about 10% of patients with coronary artery disease and that the septal thickening may either be genetically transmitted or "secondary" to the underlying cardiac disease. Further studies are planned to determine the prevalence of disproportionate septal thickening in necropsy specimens of patients dying of coronary artery disease, as well as the occurrence of disorganized cardiac muscle cells in the ventricular septae of such patients.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01659 01 CB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Echocardiographic evaluation of hypertensive patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	D.D. Savage	Clin.Associate	NHLI CB
OTHER :	J. Drayer	Research Fellow	Cardiov.Ctr.-N.Y.Hosp-Cornell Med.Ctr.
	W. L. Henry	Senior Investigator	NHLI CB
	E.C. Mathews	Spec.Fellow-Georgetown Univ.,Washington, D.C.	
	J.H. Laragh	Master Prof.Med. & Hd.Cardiov.Ctr.	N.Y.Hosp.-Cornell Med.Ctr.
	S.E. Epstein	Head,Cardiology Branch	NHLI CB

COOPERATING UNITS (if any)

Cardiovascular Center, New York Hospital-Cornell Medical Center.

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLI- Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.12	.06	.06

SUMMARY OF WORK (200 words or less - underline keywords)

Echocardiographic evaluation of patients with hypertension revealed a high prevalence of previously undetected cardiac abnormalities. Echocardiography may be more sensitive than patient history, electrocardiogram, and chest X-rays in identifying these abnormalities.

645

PHS-6040
(12-75)

Project Description: To characterize the cardiac abnormalities in hypertensive patients, 275 patients were studied by echocardiography. Hypertension was defined as a blood pressure ≥140/90. Two hundred and twenty-four patients (82%) had at least one abnormality on echocardiogram (echo). Of 244 patients with satisfactory echoes of the left ventricular septum (LVS) and posterior wall (LVPW), 130 (53%*) had concentric hypertrophy (LVS and/or LVPW ≥1.3 cm). Ten of the 244 patients (4%) had asymmetric septal hypertrophy (LVS/LVPW ≥1.3). Two of these 10 patients showed systolic anterior motion of the anterior leaflet of the mitral valve (without evidence of left ventricular outflow obstruction at rest).

Prevalence of other abnormalities included 67% (178/267) with enlarged left atrial dimension (>3.5 cm), 13% (34/264) with enlarged aortic root dimension (>3.5 cm) and 4% (9/236) with enlarged left ventricular dimension (>5.5 cm). One of these patients had a dilated cardiomyopathy with left ventricular ejection fraction of 23%. Three patients had pericardial effusions and 2 had mitral valve prolapse.

These data indicate a high prevalence of cardiac abnormalities in hypertensive patients. The majority of these patients had no cardiac symptoms, normal electrocardiograms, and normal plain chest radiographs. Hence, echocardiographic assessment appears to be an extremely sensitive technique to identify cardiac abnormalities in such patients.

* All subsequent percentages represent prevalence in population with that structure adequately defined on echo.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01660 01 CB

PERIOD COVERED
July 1, 1976 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Echocardiographic evaluation of acromegalic patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.D. Savage	Clin. Associate	NHLI CB
OTHER:	R. Eastman	Clin. Associate	NIAMDD DB
	W.L. Henry	Senior Investigator	NHLI CB
	J.S. Borer	Senior Investigator	NHLI CB
	J. Roth	Chief,Diabetes Branch	NIAMDD DB
	P. Gorden	Clin.Director	NIAMDD
	S.E. Epstein	Chief,Cardiol.Branch	NHLI CB

COOPERATING UNITS (if any)
Clinical Endocrinology Branch, NIAMDD.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLI- Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.15	.1	.05

SUMMARY OF WORK (200 words or less - underline keywords)

To better characterize the heart disease that occurs in acromegalic patients, 17 patients were studied by echocardiography. The study showed that concentric thickening of the left ventricular walls is a more common feature of acromegaly than nonconcentric thickening (in which one wall, the septum, is $\geq$ 1.3 times the thickness of the posterior wall).

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PHS-6040
(12-75)

To define the cardiac abnormalities in acromegaly, 26 patients were studied by echocardiography. The diagnosis had been established by abnormal growth hormone levels. Seventeen patients had echocardiograms (echoes) adequate to define both left ventricular septum (LVS) and left ventricular posterior wall (LVPW). One patient had asymmetric septal hypertrophy (ASH) with septum to posterior wall ratio of 1.5, without evidence of left ventricular outflow obstruction. Fourteen of these 17 patients had concentric left ventricular hypertrophy (mean LVS and LVPW thickness of 1.5 cm). Left ventricular end-diastolic dimension was normal in 15 of the 17 patients and increased (>5.5 cm) in two.

These data suggest that concentric left ventricular hypertrophy is a common feature and ASH is an uncommon feature of acromegaly. However, whether the "thickened" left ventricular wallsof such subjects have pathologic significance is unclear.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01664-01 CB

PERIOD COVERED

July 1, 1975, through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Role of the Cardiac Autonomic Nervous System in the Sudden Infant Death Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.M. Kent	Hd., Sec. Exptl. Physiology	CB	NHLI
OTHER:	B.J. Maron	Senior Investigator	CB	NHLI
	R.S. Fisher	Coroner, State of Maryland		
	S.E. Epstein	Chief, Cardiology Branch	CB	NHLI

COOPERATING UNITS (if any)

Medical Examiner's Office, Baltimore, Maryland

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

SUMMARY OF WORK (200 words or less - underline keywords)

The cardiac autonomic nervous system has been examined in infants dying of sudden infant death syndrome. Preliminary evidence appears to support the fact that the autonomic innervation of the heart is altered in at least some of the infants who die suddenly and for unexplained reasons.

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PHS-6040
(12-75)

Project Description: Sudden infant syndrome (SIDS) is the largest single cause of death between one week and one year of age in the United States. This syndrome accounts for the death of approximately 10,000 apparently well infants each year. Recent evidence from this laboratory has suggested that electrocardiographic abnormalities, namely QT interval prolongation, may be present in some infants that die suddenly. It is well known that the autonomic nervous system can influence the QT interval of the electrocardiogram. It also has been established that the autonomic nervous system is important in the electrical stability of the heart. Hence, we have examined the autonomic innervation of hearts removed from infants who have unexplained deaths as well as infants of the same age group in whom the cause of death could be specified.

The cholinergic innervation of the hearts was examined with histochemical techniques. The thiocholine precipitate method of Koelle was used for the localization of acetylcholinesterase. We have previously demonstrated that the cholinergic innervation of the sinoatrial node and atrioventricular nodes are rich in human hearts. Although cholinergic innervation of the ventricular myocardium is sparse, it is quite extensive around the ventricular conducting system. In experimental animals, we previously demonstrated that the cholinergic innervation of the ventricular conducting system is responsible for mediating the salutary effects of cholinergic stimulation on ventricular electrical stability. Thus, tissues examined were the sinoatrial node region, the interventricular septum, which included the AV node bundle of HIS and left bundle branch as well as ventricular myocardium.

To date, hearts from 13 infants between three weeks and 12 months of life have been examined. Seven of these infants died from causes that were apparent at the time of autopsy. In six of the infants, no cause of death could be found and thus the diagnosis of SIDS was made. Cholinergic activity was present in the hearts even though 24 hours had lapsed between the time of death and the time of autopsy. We found that cholinergic innervation of the hearts of infants at three weeks of age is identical to that previously demonstrated in adult humans; there is rich cholinergic innervation of the SA node, AV node and ventricular conducting system, with sparse innervation of the ventricular myocardium. In each of the seven hearts from infants dying of known causes, cholinergic innervation was intact. In one of the six infants dying of SIDS, there was no cholinergic innervation of the ventricular conducting system, although the cholinergic innervation of the atrial structures appeared intact. In the remaining five hearts of infants dying of SIDS, cholinergic innervation of all structures was intact.

There is general agreement that there is not a unique etiology for SIDS. The unexplained deaths during infancy undoubtedly contain some homicides. Furthermore, there is evidence that the pulmonary vasculature in some of the

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infants dying suddenly demonstrates arteriolar changes which appear consistent with prolonged hypoxia, presumably due to recurrent apneic episodes. Therefore, it would be unexpected to find a common mechanism of death in all infants with SIDS. The absent cholinergic innervation found in one of the hearts examined is consistent with prolongation of the QT interval, since decreasing cholinergic tone would be expected to prolong the QT interval. Absent cholinergic innervation is also consistent with ventricular electrical instability since this has been demonstrated in experimental arrhythmias.

At the present time, there are no acceptable techniques for studying the adrenergic innervation of the heart in postmortem specimens. A new technique which may provide insight into the adrenergic innervation is the use of the histochemical localization of dopamine beta hydroxylase, the enzyme which catalyzes the conversion of dopamine to norpeinephrine in the adrenergic neurons. This enzyme can be stained with immunoantibody histochemical techniques and will serve as a marker for noradrenergic neurons. In preliminary studies, it appears that this enzyme is stable in postmortem tissues. This technique would make it possible to also examine the adrenergic innervation of the hearts of infants with SIDS.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01627-02 CB

PERIOD COVERED

July 1, 1975, through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Phosphorylation of Cardiac Muscle Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.A. Lebowitz	Clinical Associate	CB	NHLI
	B. Barylko	Visiting Fellow	CB	NHLI
OTHER:	R.S. Adelstein	Hd., Sec. on Molecular Cardiology	CB	NHLI
	B.J. Maron	Senior Investigator	CB	NHLI
	J.M. Miles	Med. Biology Technician	CB	NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.7	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

Papillary muscles were attached to an isometric myograph in the presence of $^{32}P_i$. Treatment of contracting papillary muscles with norepinephrine results in a 2-fold increase in phosphorylation of myosin light chains and 4-fold increase in phosphorylation of M-line protein compared to controls. The 165,000 dalton M-line protein from dog and human hearts has been isolated and phosphorylated in vitro.

PHS-6040
(12-75)

Project Description: Using isometrically contracting cat right ventricular papillary muscles, we have undertaken to determine whether cardiac myosin and M-line protein undergo phosphorylation in vivo. To do so, we have designed and developed a life support system and isometric myograph suitable for prolonged incubation of papillary muscles in the presence of radioactive $^{32}P_i$. While incubating, the muscles are stimulated to contract twelve times per minute at the peak of their length-active tension curves. Upon cessation of incubation, muscles are rapidly frozen in liquid N_2 to prevent further metabolism. Actomyosin and M-line protein are then partially purified under conditions which inhibit further phosphorylation and dephosphorylation. We have found that both the 19,000 dalton myosin light chain and M-line protein undergo phosphorylation under our experimental conditions. To further elucidate these phenomena, we have incubated muscles in norepinephrine for several minutes prior to cessation of muscle stimulation. We have found that norepinephrine increases phosphorylation of both myosin and M-line protein. These studies suggest that phosphorylation of myosin and M-line protein are indeed relevant to myocardial function and may be important factors in controlling the strength of the heart.

Studies on phosphorylation of canine cardiac M-line protein in vitro have also been carried out. A 165,000 dalton protein has been isolated from canine cardiac muscle using two different procedures. This protein which has a S_{w20} value of approximately 5.0 is phosphorylated by an endogenous kinase. Present studies are directed toward raising an antibody to this protein so that its exact location in cardiac muscle can be determined.

Publications:

1. Elzinga M, Maron BJ, Adelstein RS: Human heart and platelet actins are products of different genes. Science 191: 94, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01644-05 CB

PERIOD COVERED

July 1, 1975, through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Phosphorylation of Myosin from Muscle and Non-Muscle Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.S. Adelstein	Hd., Sec. on Molecular Cardiology	CB	NHLI
	M.A. Conti	Chemist	CB	NHLI
	B. Barylko	Visiting Fellow	CB	NHLI
OTHER:	W. Anderson, Jr.	Chemist	CB	NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	1.8	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

The actin-activated ATPase activity of platelet myosin is under control of an enzyme, platelet myosin light chain kinase. This enzyme phosphorylates the 20,000 dalton light chain of platelet myosin. Thrombin-induced "platelet release" is accompanied by an increase in phosphorylation of the platelet myosin light chain.

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Project Description: Phosphorylation of human platelet myosin is under control of a specific protein kinase (80,000 daltons) which is dependent on Mg^{2+} but is independent of Ca^{2+} and c-AMP for its activity. Column purified myosin can be phosphorylated to approximately 1 mole/mole of 20,000 dalton light chain with a concomitant increase (7 fold) in the actin-activated ATPase activity measured at low ionic strength in the presence of Mg^{2+}. Dephosphorylation of previously phosphorylated myosin by E. coli alkaline phosphatase results in a decrease in the actin-activated ATPase activity.

In addition to the platelet myosin light chain kinase, another kinase has been identified in platelets. This second kinase is c-AMP dependent and phosphorylates f1 histone but not the myosin light chain.

Dr. James Daniel, presently working at the Hematology Laboratory, Temple University School of Medicine, Philadelphia, Pa., has continued his studies initiated in this laboratory. These studies show that platelets which have undergone a thrombin-mediated "release reaction" exhibit increased phosphorylation of the 20,000 dalton myosin light chain compared to resting controls.

Present and future studies include the following: The effect of phosphorylation will be quantitated by studying the kinetics of actin-activation of phosphorylated and non-phosphorylated myosin. Phosphorylation of the myosin fragments (heavy meromyosin and subfragment-1) will also be studied. These fragments of myosin will be prepared under two conditions: a) conditions leading to preservation of the phosphorylated site, b) those leading to destruction of the phosphorylated site. The function of the c-AMP dependent kinase will be sought, particular attention being directed at a 40,000 dalton protein which may be the endogenous substrate for this enzyme in human platelets.

Publications:

1. Adelstein, R.S.: Actin and myosin in non-muscle cells. Nature, "News and Views 255: 106, 1975.

2. Adelstein, R.S. and Conti, M.A.: Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity. Nature 256: 597, 1975.

3. Adelstein, R.S. and Conti, M.A.: The effect of phosphorylation on platelet myosin and studies on substrates for platelet myosin light chain kinase. Cold Spring Harbor Conferences on Cell Proliferation, 1976.(in press)

4. Daniel, J.L. and Adelstein, R.S.: Isolation and properties of platelet myosin light chain kinase. Biochemistry, 1976 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01645-03 CB

PERIOD COVERED

July 1, 1975, through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Contractile Proteins From Adult, Embryonic and Malignant Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Scordilis	Visiting Scientist	CB NHLI
OTHER:	R.S. Adelstein	Hd., Sec. on Molecular Cardiology	CB NHLI
	D. Henson	Staff Pathologist	NCI
	G. Cantoni	Chief, Lab. of Gen. & Comparative Biochem.	NIMH
	J.M. Miles	Med. Biology Technician	CB NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

A kinase which phosphorylates the 20,000 dalton light chain of platelet myosin has been isolated from pre-fusion rat myoblasts, human rhabdomyosarcoma cells and SV-40 transformed astrocytic neuroglial cells. The native kinase of each of these cells phosphorylates the myosin of its own cell also. These kinases differ from the myosin light chain kinase found in skeletal muscle in not requiring Ca^{2+} for activity and in having different substrate requirements.

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PHS-6040
(12-75)

Project Description: Myosin and the myosin light chain phosphorylating enzyme, myosin light chain kinase, have been isolated from four different types of cells: 1) pre-fusion rat myoblasts, 2) human rhabdomyosarcoma cells, 3) SV-40 transformed astrocytic neuroglial cells and 4) rabbit psoas muscle.

Phosphorylation of myosin was carried out either utilizing a fraction obtained following ammonium sulfate fractionation (30-55%) which contained both myosin and kinase or after Sepharose 4B column chromatography by adding the myosin and light chain kinase together in the presence of γ-labelled $AT^{32}P$ and $MgCl_2$.

These studies have shown that at least two distinct types of kinase exist. One type found in pre-fusion rat myoblasts, human rhabdomyosarcoma cells and SV-40 transformed astrocytic neuroglial cells is similar to the kinase found in platelets in not requiring Ca^{2+} for activity. Moreover, this kinase does not appear to be capable of phosphorylating skeletal muscle myosin, although the white skeletal muscle myosin kinase is capable of phosphorylating non-muscle myosin light chains.

Having demonstrated that a particular myosin kinase is associated with non-muscle cells as well as muscle cells still capable of cell division, we will study post-fusion myoblasts in an effort to see at what point in cell differentiation the kinase changes from the Ca^{2+} independent to the Ca^{2+} dependent form. Moreover, the association of the Ca^{2+} independent form of the enzyme with cells that can undergo cell division, along with evidence obtained from other laboratories indicating a role for contractile proteins in cell division, suggests that the kinase may play an important regulatory role in this process. Studies will be undertaken to determine if kinase activity varies during the various stages of cell division.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01665-01 CB

PERIOD COVERED

July 1, 1975, through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Phosphorylation and Regulation of Smooth Muscle Myosin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Chacko	Guest Worker	CB	NHLI
OTHER:	M.A. Conti	Chemist	CB	NHLI
	R.S. Adelstein	Hd., Sec. on Molecular Cardiology	CB	NHLI
	W.Anderson, Jr.	Chemist	CB	NHLI
	J.M. Miles	Med, Biology Technician	CB	NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NIH, NHLI, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1,2	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

Guinea pig vas deferans smooth muscle myosin can be phsophorylated by an endogenous kinase. The phosphorylated site is present on the 20,000 dalton light chain. Preliminary experiments indicate that phosphorylation of this smooth muscle myosin either by its own kinase or by platelet myosin light chain kinase, leads to an increase in the actin-activated myosin ATPase activity.

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PHS-6040
(12-75)

Project Description: Smooth muscle myosin has been purified from guinea pig vas deferans. The myosin has a molecular weight of 460,000 daltons and is composed of two heavy chains (200,000) and two different light chains (20,000 and 16,000). The 20,000 dalton light chain is phosphorylated by a kinase that has been separated from smooth muscle myosin by Sepharose 4B chromatography. This kinase is dependent on the presence of Ca^{2+} for its activity. Evidence for the existence of a phosphatase that rapidly dephosphorylates smooth muscle myosin has also been found.

The actin-activated myosin ATPase activity of non-phosphorylated myosin is very low. Phosphorylation of the 20,000 dalton light chain appears to increase the actin-activated ATPase activity of smooth muscle myosin. Hence, similar to the findings for phosphorylation of platelet myosin, phosphorylation of smooth muscle myosin appears to control actin-myosin interaction.

The relationship between myosin phosphorylation and actin-activation of myosin ATPase activity will be quantitated. The question of Ca^{2+} sensitivity of smooth muscle actomyosin will be investigated. At present it is not clear whether Ca^{2+} sensitivity of smooth muscle actomyosin is conferred by troponin-tropomyosin or myosin light chains (or both). One mode of Ca^{2+} control of actin-myosin interaction may be through the myosin light chain kinase, which requires Ca^{2+} for activity.

A possible connection between myosin phosphorylation and proliferation of vascular smooth muscle cells, will also be investigated. These studies will be extended to normal and diseased (atherosclerotic) aortas, in an effort to understand the mechanism of medial proliferation which is associated with atherosclerosis.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01668-01 CB

PERIOD COVERED
July 1, 1975, through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Properties of Cardiac Myosin Isolated From Embryonic, Newborn and Adult Chicken Hearts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.A. Lebowitz	Clinical Associate	CB	NHLI
OTHER:	R.S. Adelstein	Hd., Sec. on Molecular Cardiology	CB	NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Cardiology Branch

SECTION
Molecular Cardiology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	-

SUMMARY OF WORK (200 words or less - underline keywords)

Cardiac myosin was isolated from chickens of the following ages: 2-5 days and 14-16 days post-fertilization, newborn and adult. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate or 8M urea (pH 8.6) showed no differences in heavy or light chains of myosin. There was no difference in the K^+-EDTA activated ATPase activity.

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PHS-6040
(12-75)

Project Description: Although differences in myocardial contractile proteins among species have been shown, no study has been performed to delineate variation within a single species as a function of age and development. We studied myosin from white leghorn chick embryo and chicken hearts of the following ages: 1) 2-5 days post-fertilization, 2) 14-16 days post-fertilization, 3) newborn and 4) adult chicken hearts. Hearts were removed and dissected as free as possible of epicardial fat, valves, blood and great vessels. Tissue so obtained was subjected to standard techniques for the purification of myosin. Myosin so obtained was subjected to polyacrylamide electrophoresis in gels containing either 1% sodium dodecyl sulfate or 8M urea (pH = 8.6). Specific K^+-EDTA ATPase activity and actin-activated ATPase activity were also determined. We found that regardless of age or developmental stage, chicken cardiac myosin had a K^+-EDTA specific activity of .6 µm/mg/min . and an actin activated specific activity of .2 µm/mg/min. Also, myosin appears to contain only one heavy chain type and two light chain types which migrate on acrylamide gels without change in either apparent molecular weight or net charge at all ages studied. We concluded that so far as these studies were concerned, cardiac myosin undergoes no changes from the cardiac loop stage to adulthood.

Publications: None

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

The work of the Hypertension-Endocrine Branch has been concerned primarily with factors known or thought to be related to the control of blood pressure. Such factors include:

1) Secretion of neurotransmitters and their role in hypertension in rat and man.

2) Prostaglandins and their role as vasodepressors; the role of prostaglandin synthetase inhibitors.

3) The kallikrein-kinin systems

4) The renin-angiotensin-aldosterone system and its relation to prostaglandins and to hypertension.

Pharmacologic and clinical studies were done with agents known to influence blood pressure, e.g. (1) aminoglutethimide, (2) triamterene, (3) spironolactone, and (4) propranolol.

1) Secretion of neurotransmitters and their role in hypertension in rat and in man:

The three mixed-function oxygenases that catalyze important steps in the synthesis of norepinephrine, dopamine, and serotonin, respectively - tyrosine hydroxylase, dopamine beta hydroxylase and tryptophane hydroxylase, were studied *in extenso*. Tyrosine hydroxylase has been purified to a degree ten times that previously accomplished. Dopamine beta hydroxylase, shown to be a tetrameric glycoprotein was found to have four identical subunits, each containing one copper molecule, and to have a dimeric, active form. The results indicate that the substrate reacts directly with the copper-containing active sites. Tryptophane hydroxylase was further purified, but the lability of the isolated enzyme has limited attempts to prepare the pure enzyme. Tyrosine hydroxylase and tryptophane hydroxylase, which regulate the biosynthesis of norepinephrine and serotonin, respectively, were found to have a common reaction mechanism requiring tetrahydrobiopterin as cofactor.

It has been shown that a protein phosphorylated by a cAMP-dependent kinase probably activates tyrosine hydroxylase by decreasing the Km of the enzyme for its cofactor.

This phosphorylated protein can be partially purified on Sephadex, and retains its ability to activate the enzyme. Activation of the enzyme by dopamine-receptor blockade - e.g., by haloperidol - probably involves such a phosphoprotein, as does its activation by micelles of phospholipid or by heparin.

Studies on the regulation of tryptophane hydroxylase suggest that end-product inhibition probably does serve to limit synthesis of this enzyme. It was found that p-chloramphetamine, which is specifically taken up by serotonergic nerve endings, causes marked loss of tryptophane hydroxylase in these nerve endings. A number of studies were done on the neuronal control of protein

synthesis: this included the trans-synaptic induction of tyrosine hydroxylase in sympathetic ganglia and the carotid body, the beta-receptor-dependent induction of serotonin-N-acetyl-transferase in the pineal, and the sympathetic-dependent synthesis of non-collagen protein in the mesenteric arteries.

The trans-synaptic induction of tyrosine hydroxylase in sympathetic ganglia was found to be greatly potentiated by carbohydrate-active adrenal steroids, and to be blocked by the inactive steroid analog, 11-desoxy cortisol; it was found to require also activation of nicotinic receptors. It was found that the tyrosine hydroxylase in the carotid bodies, which produce dopamine, can be induced by hypoxia within 24 hours and requires new protein synthesis. Hypoxia was found to be without effect on the tyrosine hydroxylase of adrenal medulla or sympathetic ganglia.

The beta-receptor-dependent regulation of serotonin N acetyl transferase (NAT) in the pineal gland was further investigated. It was found that induction of the protein (by beta receptor activation) continued despite inhibition of messenger-RNA synthesis by 80 per cent (with cordycepin) although it was prevented by inhibition of all mRNA synthesis (with Actinomycin D). The results suggest an atypical 'message' for NAT, or some translational control of its synthesis.

The study of sympathetic-dependent control of non-collagen synthesis in arteries was continued with spontaneously hypertensive Okamoto rats (SHR). Previous studies had shown that these rats produce such protein (labeled by lysine incorporation) more rapidly than do normotensive control rats. Moreover, control of blood pressure by hydralazine does not lower the rate of synthesis, but sympathectomy or sympathetic blockade does - results which indicate that this constitutes another form of sympathetic nerve-dependent protein synthesis. SHR and stroke-prone SHR were found to have significantly more tyrosine hydroxylase in the hypothalamus than normotensive controls, and to have higher plasma norepinephrine and DBH at three weeks of age.

2) Prostaglandins and their role on vasodepressors; the role of prostaglandin synthetase inhibitors:

Prostaglandins were studied in isolated renal medullary interstitial cells, for their effects upon intraarterial and intravenous injection, and, in man, for the effects of their withdrawal by synthetase inhibition.

Isolated renal medullary interstitial cells from the rabbit were grown in tissue culture and shown to produce prostaglandins E (PGE) and prostaglandins F (PGF) as measured by radioimmunoassay. Production of PGE by these cells was stimulated with arachidonic acid, bradykinin, angiotensin II, vasopressin and hypertonic buffer alone; it was inhibited with potassium, acetyl salicylic acid, indomethacin, ibuprofen, and meclofenamic acid. Renal PGE excretion was shown to have a prominent circadian rhythm with acrophase at about 1900; this curve parallels that for urinary potassium. Urinary immunoreactive PGE (iPGE) decreases with assumption of the upright posture.

With infusion of saline in the dog, urinary iPGE decreases throughout the morning. This result suggested that the decrease of renal PGE might be playing a part in natriuresis, since renal medullary PG's have been shown to increase reabsorption of soium (cf. 1974 Annual Report). In fact, however, the same decline in iPGE with time occurred, without sodium diuresis, when dextrose-water was infused during the same morning hours. Thus the decline represents part of the circadian pattern of PGE excretion in the dog.

Prostaglandins of the E series, infused into the renal artery of dogs, were shown to stimulate production of renin by the injected kidney but not the contralateral one. Conversely, indomethacin so infused promptly decreased the secretion of renin. Thus PGE's appear to constitute an important agent for control of renin secretion. In the syndrome of juxtaglomerular hyperplasia, with hyperreninemia, aldosteronism and hypokalemic alkalosis ("Bartter's syndrome"), urinary PGE's were found elvated by gas chromatography mass spectrometry with tetradeuterated internal standard. Treatment with indomethacin, lowered urinary PGE by over 50 per cent and temporarily corrected the hyperreninemia, th aldosteronism and the hypokalemic alkalosis. These results suggest that renal PGE's play an integral part in the mechanism of the pathophysiologic changes in the syndrome.

3) The kallikrein-kinin systems.

These consist of (1) a plasma system of prekallikrein, kininogen and bradykinin, in which prekallikrein is activated to kallikrein by activated Hageman factor, after which it can release bradykinin from kininogens. (This system is further complicated by the requirement of high-molecular-weight kininogen for optimal activation of Hageman factor.) The bradykinin, in turn can be inactivated by kininases, of which converting enzyme has been most studied, and (2) a "glandular" system, wherein kallikreins produced in salivary glands and kidney, may react with kininogen to release lysyl bradykinin. Whereas no function of urinary kallikrein has been established, it may react with kininogen in kidney tubules to produce lysyl bradykinin, which may affect tubular permeability.

Plasma prekallikrein concentration was found to be elevated in patients with primary aldosteronism and in some patients with Bartter's syndrome; it was normal in patients with idiopathic hypertension and was not changed by changes in posture or sodium intake in normal subjects or in subjects with idiopathic hypertension. Such subjects did show a significant circadian rhythm.

In spontaneously hypertensive rats, plasma prekallikrein was found to be unaffected by sodium intake, but to increase gradually with age. When the rats reached the stage of severe hypertension, plasma prekallikrein decreased significantly. In normal rats, combined treatment with desoxycorticosterone (DOCA) and salt increased plasma prekallikrein, and this effect was less marked when the rats became hypertensive. Thus the changes in plasma prekallikrein in

the rat closely parallel those previously reported in urinary kallikrein in man, which also increases with sodium-retaining steroids, and decreases with increasing blood pressure. Plasma kininogen was studied in normal human subjects, and found to be unaffected by changes in posture or sodium intake. It was elevated in patients with Bartter's syndrome.

Plasma bradykinin which rises with upright posture and sodium restriction, was found to be elevated in patients with Bartter's syndrome. (This finding is of interest, since the blood pressure remains normal in this syndrome despite elevation of plasma angiotensin II.)

Urinary kallikrein, previously reported to increase with sodium-retaining steroids (e.g., in primary aldosteronism) and to decrease in idiopathic hypertension, was found elevated in patients with Bartter's syndrome, despite low values for urinary kinin (lysyl bradykinin).

When prostaglandin inhibitors were given to patients with Bartter's syndrome, the plasma bradykinin and the urinary kallikrein returned to normal as did plasma renin, aldosterone excretion and plasma potassium (vide supra).

In scleroderma urinary kallikrein was low, as was renal blood flow: this suggests a relation between the two, as is found in dogs with renal artery constriction.

Urinary urokinase (an enzyme found in urine which can cleave plasma plasminigen to plasmin) was studied in normal subjects. It was found to increase with the upright posture but not with sodium restriction or potassium loading.

Uropepsin and its substrate urokininogen appear in urine; their interaction produces methionyl-lysyl-bradykinin (met-lys-bk). Since addition of pepstatin to urine prevents release of met-lys-bk, it is thought that the met-lys-bk is produced only in the urine, thus representing an event of no physiologic importance.

4) The renin-angiotensin system and its relation to prostaglandins and to hypertension.

The relationship of the PGE's to renin production has been mentioned. In view of the established dependence of renin release upon beta-adrenergic stimulation, the beta receptor blocker, dl propranolol, was studied in dogs and in human subjects with hypertension.

In conscious dogs not previously depleted of sodium, dl propranolol decreased plasma renin activity without affecting blood pressure, plasma renin concentration, plasma potassium or packed cell volume. Heart rate (and thus presumably cardiac output) decreased as plasma renin decreased. The results thus suggest a beta-receptor control of renin release even under "unstimulated" conditions.

The "renin status" of hypertensive patients was determined from simultaneous measurement of plasma renin activity, urinary aldosterone and urinary sodium. In such a profile, a number of subjects appeared to have "low-renin" hypertension, and others to have "high-renin" hypertension; these groups were further studied.

It was found that most of the patients to fall into these categories were later rejected by more stringent criteria. "Low-renin hypertension" was re-defined as hypertension in which plasma renin remained below 2.5 ng Angiotensin II formed per ml per hour after the patient had come into sdium balance on a low-salt diet begun with furosemide, 120 mg a day.

As regards "high-renin hypertension", the selection of criteria for this diagnosis could not be clarified without a "suppression test" the rapid infusion of 2000 ml of normal saline, after which PRA was below 7.5 in normal subjects, but above this value in patients diagnosed as having "high-renin hypertension".

Clinical screening of patients was followed by the extensive use of experimental treatment protocols. The studies with aminoglutethimide, triamterene, and spironolactone are described at length. Aminoglutethimide (alpha-para-amino phenyl-alpha-ethyl glutarimide) decreases adrenal secretion of aldosterone and cortisol; with increase of plasma ACTH, the effect on cortisol is lost, and only zona glomerulosa steroidogenesis is inhibited. (Increase of plasma renin activity will not overcome the block.) In patients with low-renin hypertension, suspected of secreting an unidentified sodium-retaining steroid, aminoglutethimide lowered blood pressure and aldosterone secretion. It was shown to have no effect on secretion of 18-OH-desoxycorticosterone (cf. results of Melby et al) or 16-beta-OH-dehydroepiandrosterone (cf.results of Liddle et al) thus making a role for these steroids in the elevation of blood pressure in these patients extremely unlikely.

To explore further the possible role of an unidentified sodium-retaining steroid in the patients with low-renin hypertension, a cooperative study was set up with University of California at San Francisco (Dr. John Baxter).

Plasma of patients is assayed for ability to displace 3-H aldosterone from its receptor site in rat kidney slices. The assay, which can detect the rise in plasma aldosterone produced by two hours in the erect position, detects most sodium-retaining steroids at concentrations inversely proportional to their sodium-retaining ability. It has been applied to plasma of patients with low-renin hypertension. Three patients have thus been found whose plasma contains an unidentified substance that binds to these "mineralcorticoid" receptors. The great majority of patients with low-renin hypertension have given negative results that is, plasma aldosterone accounts for all the binding activity. The steroid 16-beta-hydroxy-dehydroepiandrosterone, thought to play a role in some patients with low-renin hypertension, does not bind to the receptors. Thus if it has sodium-retaining properties, this action is not transduced through aldosterone binding sites.

Triamterene (2,4,7 triamino-6-phenylpteridine) is being assessed as an anti-hypertensive agent which prevents potassium loss, in the hope that it will have less side-effects than aldactone. Whereas the effect of the drug was to lower blood pressure, and to restore plasma potassium to normal if it had been lowered by diuretic therapy, it frequently lowers the filtration rate, and infrequently produces hyperkalemia or skin rash.

Studies with spironolactone (17-hydroxy, 7-mercapto-3-oxo-17-alpha-pregn-4-ene 21-carboxylic acid lactone 7-acetate) have been continued. The drug has antihypertensive effects in "low-renin" and in normal-renin idiopathic hypertension as well as in that of primary aldosteronism resulting from tumor or adrenal cortical hyperplasia. Gynecomastia and loss of libido in males has not been explained. Whereas spironolactone transiently lowers plasma testosterone, this is followed by rise of plasma LH, and plasma testosterone returns to normal. Plasma progesterone and 17-OH-progesterone rise and remain elevated with therapy. As this occurs despite return of plasma LH to normal, it probably depends in part upon the action of spironolactone in blocking adrenal and testicular C17-20 desmolase which in turn depends upon destruction of adrenal P450. Spironolactone increases plasma renin activity in all patients but this returns to normal in all patients by the fourth week after the drug is stopped.

Propranolol (1 isopropylamino 3 inaphthyloxy propanol) was found to lower blood pressure in all types of hypertension. As it is virtually without side effects in patients without cardiac failure, causing only fatigue at high doses, it is being studied for dosage schedules required to give desired blood concentrations.

Studies involving pituitary-adrenal-gonadal interactions were initiated. In a patient with congenital adrenal hyperplasia and a testicular tumor, the testicular size was shown to increase with injections of ACTH and to be suppressed with dexamethasone. The values for plasma cortisol from the spermatic vein were three times those from the peripheral veins. This shows that the testicular tumor secreted cortisol, despite the morphologic resemblance of the Leydig cells. Children with congenital adrenal hyperplasia were studied to ascertain if nocturnal injections of dexamethasone would be beneficial in maintaining a normal growth pattern in these patients. The dexamethasone could suppress adrenal androgens, which are usually effective in promoting the premature closure of epiphyses with resultant short stature. The administration of dexamethasone at night could, however, be ineffective if, at the same time, it inhibited the nocturnal secretion of growth hormone.

Clinical studies on the control of parathyroid hormone secretion reveal that nephrogenous cAMP provides a sensitive and specific measure of parathyroid function in man. In a group of 58 patients with primary hyperparathyroidism, immunoreactive parathyroid hormone (iPTH) was elevated in 71% and nephrogenous cAMP was elevated in 90%. The methodology

for determining iPTH is limited in that there is heterogeneity of the circulating forms of the circulating protein hormone and no one assay accounts for all of the hormone. Nephrogenous cAMP has been useful in developing diagnostic criteria for diagnosis of patients with hypercalciuria of other etiologies. Patients with absorptive hypercalciuria have normal basal parathyroid function and the nephrogenous cAMP is suppressed by more than 50% after oral doses of calcium; patients with "renal leak" hypercalciuria have elevated basal parathyroid function, with only slight suppression of the nephrogenous cAMP after oral doses of calcium. A definitive diagnosis of primary hyperparathyroidism, absorptive hypercalciuria or renal hypercalciuria can be made by the measurement of nephrogenous cAMP as an index of parathyroid function.

Other studies involved in the production of hypercalciuria include dietary protein and pharmacologic agents such as aminoglycosides. Normal subjects, patients with nephrolithiasis and patients with osteoporosis were found to double their urinary calcium when ingesting a high protein diet (2.0 gm/kg/d) in contrast to that during control periods on a low-protein diet (0.5 gm/kg/d). It is thus apparent that excessive dietary protein may be a factor in promoting negative calcium balance in patients with osteoporosis. Although the administration of the aminoglycosides, gentamycin and kanamycin, in large doses to laboratory rats produced increased urinary calcium, acute injections into normal subjects at therapeutic dosages did not produce hypercalciuria.

Prostaglandins have been implicated in the hypercalcemia of some forms of carcinoma. Accordingly, prostaglandin E_1 was studied for its effects on protein and glycoprotein metabolism in rat parathyroid glands *in vitro*. Concentrations of PGE_1 of 100 ug/ml in the medium produced significant decreases in the incorporation of radioactive uridine and thymidine into RNA and DNA, respectively, and inhibited the secretion of immunoreactive parathyroid hormone. Whether the prostaglandins have an effect on parathyroid function *invivo* remains to be determined.

Phosphoethanolamine excretion has previously been considered pathognomonic for the genetic disorder hypophosphatasia. Studies in this laboratory reveal that variations in normal diet and physiology can significantly alter its excretion. Increased amounts of phosphoethanolamine has been found in the urine of patients with various bone diseases, hypertension, and various endocrine disorders, findings which suggest that a fundamental process common to many diseases results in the increased urinary loss.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01801-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Outpatient Hypertension Diagnostic Screening Program

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Taylor	Clinical Associate	HE NHLI
OTHER:	W. R. Snodgrass	Guest Worker	CP NHLI
	D. E. Rollins	Research Associate	CP NHLI
	R. A. Rosen	Clinical Associate	KE NHLI
	J. R. Gill, Jr.	Senior Investigator	HE NHLI
	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI
	C. S. Delea	Administrative Assistant	HE NHLI
	J. W. Cox	Biochemist (Supervisor)	HE NHLI

COOPERATING UNITS (if any) Section on Clinical Pharmacology, NHLI, NIH

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Random renin-sodium profiling produces a heterogeneous population of low renin essential hypertensive (LREH) patients, many of whom would not be classified as LREH on repeat examination. The stimulus of a 9 mEq Na diet apparently yields a more homogeneous population of patients with LREH. Forty mg of furosemide is an insufficient stimulus to identify patients with LREH.

671

PHS-6040
(12-75)

Project Description:

The application of sophisticated methods for measuring renin and aldosterone to the study of hypertensive patients has contributed to our ability to diagnose secondary forms of hypertension such as primary aldosteronism and renal artery stenosis. Several procedures for measuring renin and aldosterone in hypertensive patients under defined physiologic and/or pharmacologic conditions have been proposed.

Objective: The purpose of this study is to evaluate the accuracy of these various methods for categorizing hypertensive patients into selected subgroups based on their renin and aldosterone response to a defined stimulus. Currently the most commonly used method for such categorization relates plasma renin activity after 3 hours of upright posture to the individual's 24 hour urinary sodium and aldosterone on a random diet, the "renin-sodium-aldosterone index". Other investigators have classified hypertensive patients according to their renin response to upright posture and variable oral dosages of furosemide. Still others have examined the renin and aldosterone response to upright posture in patients who have achieved sodium balance on a low sodium diet.

Project Protocol: Each patient referred to the Hypertension outpatient clinic is seen by a physician who takes a history and performs a physical examination. A chest x-ray, electrocardiogram, urinalysis, urine culture and routine serum, chemistries are obtained during the patient's first visit. An intravenous pyelogram and radioactive renogram are obtained between the first and second visits. Each patient is taught to take his own blood pressure and is requested to take it 6 times a day. On the morning of the second visit, the patient brings a 24 hour urine sample for sodium, potassium, creatinine, 17OHCS, 17KS and aldosterone excretion rate, and 45 min.-supine and 3 hour- upright blood samples are obtained for plasma renin activity, aldosterone and cortisol. The same protocol is followed on the third visit as the second except that each patient is given Lasix 40 mg after the first blood sample is obtained. Based on these data the patients is then placed in a diagnostic category and he is included in one of several ongoing protocol studies if appropriate. Patients presumptively classified as having low renin essential hypertension, based on their renin-sodium-aldosterone index, are admitted to the NIH Clinical Center and allowed to achieve sodium balance on a 9 mEq sodium diet. Three hour upright plasma renin activity, plasma aldosterone and plasma cortisol are then measured.

Normotensive volunteers giving informed consent are studied in a similar fashion except that they are not given Lasix.

Major Findings: We have evaluated 150 hypertensive patients by this method and compared their values with the renin-sodium-aldosterone index in 50 normotensive subjects. LREH with normal plasma and urinary aldosterone was found in 40% of these patients. One hundred and one were re-examined following 40 mg of furosemide orally and 30 of these (29%) still met the criteria for a

diagnosis of LREH. The renin system was further evaluated in the hospital in 19 of these 30 patients after they had achieved balance on a 9 mEq sodium diet. Only 8 of the 19 had μPRA $\leq$ 2.5 and could still be classified as LREH. During this study additional random determinations of μPRA related to sodium excretion were made in a few patients; of interest was the observation that 5 of 19 patients originally classifed as LREH by the renin-sodium index, but not by the 9 mEq Na diet, had μPRA > 2.5 on repeat random examination. Conclusions: 1) Random renin-sodium profiling produces a heterogeneous population of LREH patients, many of whom would not be classified as LREH on repeat examination; 2) the stimulus of a 9 mEq Na diet apparently yields a more homogeneous population of patients wtih LREH; 3) forty mg of furosemide is an insufficient stimulus to identify patients with LREH.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01803-02 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Aminoglutethimide in Low-Renin Essential Hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.R. Mitchell	Chief, Sec. on Clin.Pharm.	CP NHLI
OTHER:	A.A. Taylor	Clinical Associate	HE NHLI
	R.B. Franklin	Visiting Fellow	CP NHLI
	J.R. Gill, Jr.	Senior Investigator	HE NHLI
	W.R. Snodgrass	Guest Worker	CP NHLI
	R.J. McMurtry	Clinical Associate	CP NHLI
	R.A. Rosen	Clinical Associate	KE NHLI
	F.C. Bartter	Chief, Hyper.-Endo. Br.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

SUMMARY OF WORK (200 words or less - underline keywords)

The blood pressure lowering effect of aminoglutethimide (AG) (1 gm/day for 2 weeks) was compared with its effect on the major pathways of adrenal steroid biosynthesis in 4 patients with LREH and in 2 patients with normal renin essential hypertension (NREH). AG reduced BP in all patients, and decreased the excretion of aldosterone 75%, but failed to decrease plasma concentrations of progesterone, 17-hydroxyprogesterone, deoxycorticosterone, 11-desoxycortisol, or cortisol, or the urinary excretion of 16-beta-OH-dehydroepiandrosterone, 17-hydroxy or 17-ketosteroids. Secretion rates for 16-beta-OH DHEA were not changed by AG. BP-lowering action of AG in LREH probably resulted from inhibition of aldosterone synthesis; 16-beta-OH-DHEA is unimportant in LREH because its secretion is not reduced when BP is lowered by AG; Na^+-retaining steroids other than aldosterone are not important in LREH because no other major steroid pathway was inhibited by AG; AG altered steroid synthesis similarly in 2 normal-renin hypertensive patients but lowered BP less possibly because their plasma renin during AG was 5 times greater than that of LREH patients.

PHS-6040
(12-75)

Project description:

Objectives: Patients were tested for the blood pressure-lowering effect of aminoglutethimide, an inhibitor of adrenal steroid synthesis. The pathways of adrenal steroid synthesis that are inhibited by aminoglutethimide in blood-pressure responsive patients, and therefore the pathways that might be mediating the hypertension, will be determined.

Treatment with aminoglutethimide lowers the blood pressure of patients with hypertension secondary to primary aldosteronism or Cushing's syndrome (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endo. Metab. 27: 481, 1967; and Gorden, et al., J. Clin. Endo. Metab. 28: 921, 1968). In 1969 Liddle's group (Woods, et al., Arch. Int. Med. 123: 366, 1969) reported that 6 of 9 hypertensive patients with the syndrome of low-renin (normal aldosterone) essential hypertension experienced a lowering of blood pressure after administration of aminoglutethimide but patients with normal renin hypertension failed to respond. They noted also that the hypertension of low-renin patients responded to therapy with spironolactone (Carey, et al., Arch. Int. Med. 130: 849, 1972), a renal antagonist of sodium-retaining steroids, and to bilateral adrenalectomy (Gunnels, et al., Ann. Int. Med. 73: 901, 1970). This report triggered a widespread search for the excessive adrenal secretion of a sodium-retaining steroid other than aldosterone, and several groups subsequently have postulated that various steroids are of etiologic significance in low-renin hypertension (Brown, et al., Lancet ii: 243, 1972; Melby, et al., Circ. Res. 23: 11-143, 1971; Kuchel et al., Circ. Res. 23: 11-150, 1971; Messerli, et al., Proc. 56th Meeting of Endocrine Society, p. A-63, 1974; Slaton, et al., Clin. Res. 23: 45A, 1975; and Hisahsatu, et al., J. Clin. Endo. Metab. 40: 156, 1975. However, in these studies only the particular steroid of interest was examined. No attempt was made to determine secretion rates of most other adrenal steroids nor to demonstrate cause-effect relationships between increased steroid secretion and elevation of blood pressure. Indeed, the direct involvement of the proposed steroids in the pathogenesis of low-renin hypertension can be questioned, since none of the steroids have has sufficient intrinsic sodium-retaining activity to be physiologically effective in the amounts apparently secreted by patients with low-renin hypertension.

The antihypertensive effects of aminoglutethimide result from its inhibition of adrenal steroid secretion and the accompanying renal loss of sodium and water; the drug has no direct effect on the kidney, the adrenergic nervous system or vascular smooth muscle in adrenalectomized animals and people (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; Woods, Liddle, et al., Arch. Int. Med. 123: 366, 1969). Thus, the secretion of a sodium-retaining adrenal steroid, be it aldosterone or an unidentified steroid, should play an important pathogenetic role in the hypertension of low-renin patient whose blood pressure responds to aminoglutethimide therapy.

Careful review of numerous biochemical studies in dogs and other animals, with sufficient clinical studies to confirm that man responds similarly, reveals that the secretion of cortisol by the adrenal zona fasciculata is only minimally affected by aminoglutethimide in endocrinologically normal subjects because of a compensatory increase in ACTH secretion (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; and Woods, Liddle et al., Arch. Int. Med. 123: 366, 1969). In contrast to the cortisol pathway, secretion of aldosterone by the adrenal zona glomerulosa remains inhibited by aminoglutethimide even after months of continuous therapy and compensatory increases in renin do not overcome the inhibition (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; and Woods, Liddle et al., Arch. Int. Med. 123: 366, 1969).

This phenomenon of preferential inhibition of aldosterone synthesis provides a powerful tool for defining the physiologic role of the steroids postulated as having etiologic significance in the genesis of low-renin hypertension. If the secretion of a particular steroid is not inhibited by aminoglutethimide therapy when blood pressure is reduced, then that steroid cannot be solely responsible for the hypertension. By the same rationale, one can determine which adrenal steroid synthetic pathways are inhibited by aminoglutethimide in blood pressure-responsive patients and therefore which pathways might be mediating the hypertension.

Methods Employed: Patients with low-renin hypertension on a normal diet were hospitalized for 3 days and secretory rates were determined by double isotope dilation assay following intravenous administration of radioactive tracer doses of ^{3}H-16-beta-hydroxydehydroepiandrosterone (1 µg,4 µCi), and ^{3}H-18-hydroxydesoxycorticosterone (1 µg, 4 µCi). Five plasma samples (10 ml each) were obtained, and urine was collected for 48 hours. Plasma DOC, desoxycortisol, cortisol, aldosterone, progesterone and 17-alpha-hydroxy-progesterone were determined by radioimmunoassay and urinary excretion of metabolite of cortisol and aldosterone were assayed chemically or by radio-immunoassay.

Following these control determinations, patients were treated with amino-glutethimide (1 g per day in divided doses) and steroid determinations were repeated after 4 days of treatment. Patients were then discharged and followed weekly in the clinic for another 14 days. As outpatients, they recorded their blood pressure several times daily under the normal conditions of their environment. At each weekly clinic visit, blood (20 ml) was obtained for electrolytes, CBC, SGOT, flucose, urea nitrogen, aldosterone, cortisol and renin. Patients were then hospitalized for a final 3 days and the steroid secretory and excretory determinations repeated exactly as above (total duration of treatment with aminoglutethimide = 3 weeks).

Major Findings:

Aminoglutethimide (AG) has been administered to 6 patients classified as having low renin hypertension (LREH) by the renin-sodium index. Each of the patients had normal values for plasma and urinary aldosterone. Two of the 6 patients subsequently had a normal plasma renin response after 3 hours of upright posture while in balance on a 10 mEq sodium diet and have been classified as normal renin hypertension (NREH). Three patients were treated for 3 weeks with aminoglutethimide while in 3 patients drug treatment was discontinued after 7-10 days due to the development of intolerable side effects such as total body skin rash and fever.

The following data have been obtained.

	Hi Na diet		Hi Na+AG		Lo Na diet	
	LREH	NREH	LREH	NREH	LREH	NREH
Mean BP(mmHg)	120±7[a]	117±2	94±1*	111±1	111±2	112±2
Up PRA(ng/ml/hr)	0.5±0.1	2.2±0.2	1.3±0.4	7.9±1.5	1.4±0.4	9.2±1.8
$U_{Na}V$(mEq/24 hr)	196±22	221±43	188±15	230±34	13±3*	7±2*
Aldosterone Excretion Rate (ug/24 hr)	4.1±0.7	4.6±0.6	0.9±0.2*	0.8±0.2*	10.7±1.4*	14.9±1.7*

a = mean ± SEM

* = significantly different from values on high sodium diet ($P < .05$)

Aminoglutethimide significantly lowered blood pressure and decreased the plasma and urinary values of aldosterone but failed to reduce the plasma levels of cortisol, progesterone, 17-hydroxyprogesterone, 11-deoxycortisol, deoxycorticosterone or urinary excretion of total 17-ketosteroids, 17-hydroxysteroids, or 16-beta-OH-dehydroepiandrosterone when compared with pretreatment values. Secretion of 16-beta-OH-DHEA was also unchanged by AG treatment.

Significance:

The blood-pressure-lowering action of AG in LREH probably resulted from inhibition of aldosterone synthesis. 16-beta-OH-DHEA appears unimportant in maintaining the hypertension of LREH because its secretion is not reduced when blood pressure is lowered by AG. Sodium-retaining steroids other than aldosterone are not important in LREH because no other major steroid pathway was inhibited by AG. AG altered steroid biosynthesis similarly in 2 patients with NREH, but lowered their blood pressure less, possibly because their PRA during AG was 5 times greater than that of LREH patients.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01804-02 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effects of Spironolactone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Taylor	Clinical Associate	HE NHLI
OTHER:	J. R. Mitchell	Chief, Sec. of Clin. Pharm.	CP NHLI
	W. R. Snodgrass	Guest Worker	CP NHLI
	D. E. Rollins	Research Associate	CP NHLI
	R. A. Rosen	Clinical Associate	KE NHLI
	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any)

Section of Clinical Pharmacology, NHLI, NIH

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of spironolactone (S) on renin and adrenal and gonadal steroids measured in normal renin (NREH) and low renin (LREH) hypertensive patients before, during (2,4,8 and 12 weeks), and after (2 and 4 weeks) therapy with S at dosages ranging from 150 to 400 mg/day. Blood pressure was significantly lower in LREH than in NREH after 12 weeks of S therapy. Upright plasma renin activity (uPRA) rose in both groups and had decreased to pretreatment values in within 4 weeks of stopping S. Plasma progesterone and 17-OH progesterone were 2-4x greater during S therapy than in the pre-or post-therapy periods in all patients. In contrast, plasma testosterone, estradiol, FSH, LH, prolactin, and urinary 17-hydroxycorticosteroids and 17-ketosteroids did not change with S. In 2 menopausal women with undetectable estradiol, S-induced changes in plasma progesterone and 17-OH progesterone were not significantly different from those of other patients. Two of 6 men developed gynecomastia and 1 of 4 women had irregular menses. Patterns of plasma and urinary steroids in these patients were not different from those of patients without clinical side-effects. We conclude that the hyperreninemia induced by S does not persist for longer than 4 weeks after stopping S. S-induced changes in the concentrations of sex steroids (which are probably of adrenal origin) can neither predict nor account for the developement of endocrine side-effects during S therapy.

PHS-6040
(12-75)

Project No. Z01-HL-01804-02 HE

Project Description:

Objectives: To examine the effects of spironolactone or renin-angiotensin-aldosterone physiology and on sex steroids in hypertensive patients.

Spironolactone is widely used as a potassium-conserving diuretic and antihypertensive agent. It has been suggested by some as the drug of choice in patients with low renin essential hypertension, a subgroup comprising approximately 25% of the hypertensive population. Previous work by some of us has shown that spironolactone destroys cytochrome P-450 in the adrenal and testis of certain experimental animals (Menard et al., Endo. 94: 1628, 1974; Menard et al., J. Steroid Biochem. 5: 365, 1974). Cytochrome P-450 is a co-enzyme necessary for normal function of the steroid 17-hydroxylase enzyme; 17-hydroxylase activity is decreased in experimental animals given spironolactone. A recent study of the effect of 400 mg spironolactone per day for five days in normal male volunteers showed only a transient rise in plasma LH and FSH but no change in plasma testosterone, estradiol, or prolactin. These findings failed to explain the frequently observed side effects of gynecomastia, decreased libido, and impotence in male hypertensives taking spironolactone.

Significant interference by spironolactone with parameters of the renin-angiotensin-aldosterone system in low renin hypertensive patients is demonstrated by the recent report of Lowder etal., Liddle (NEJM 29: 243, 1974), that the increased upright plasma renin activity induced by spironolactone in low renin hypertensive patients persisted for 13-36 weeks after discontinuation of the drug.

Methods: Patients previously classified as having essential hypertension with normal or suppressed plasma renin activity by diagnostic studies performed while off all antihypertensive medications are considered for inclusion in the study. Patients with prior history of malignant hypertension, evidence of cardiovascular compromise or severe impairment of renal function (creatinine clearance = 70 cc/min or less) are excluded. All patients are provided with equipment and taught to take their blood pressure. Blood pressures are recorded both by the patient at home and by physicians in the outpatient clinic throughout the study.

Following pretreatment measurements of the plasma and urinary parameters listed below, each patient received spironolactone, 400 mg/day, for a maximum of 12 weeks. After 12 weeks of therapy, spironolactone was discontinued and the patient observed for an additonal 4 weeks before reinstitution of antihypertensive therapy unless development of signs and symptoms of cardiovascular compromise or accelerated hypertension necessitate removal from the study and treatment at an earlier time. Before, and at regular 2-4 week intervals during and after discontinuation of spironolactone, the following plasma and urinary measurements are made: Plasma renin activity, aldosterone, cortisol, deoxycorticosterone, 11-deoxycortisol, testosterone, progesterone, 17-hydroxyprogesterone, 17β-estradiol, prolactin,

LH, FSH, Na, K, Cl, CO_2, BUN, creatinine, sex hormone binding globulin; urinary creatinine, Na, K, aldosterone, 17-hydroxycorticosteroids, 17-ketosteroids, and aldosterone excretion rate.

Major Findings: Five patients with NREH and 5 with LREH have been studied. On S-therapy 2 of the 6 men developed gynecomastia and 1 of the 4 women had irregular menses. The study was not completed in 4 patients, 1 because of the development of a drug rash and 3 because of the occurrence of intolerable side-effects such as gastrointestinal upset or muscle cramps.

Pretreatment blood pressures (BP) in the 2 groups were not different but BP in LREH was significantly lower than in NREH after 12 weeks of S therapy. Upright plasma renin activity, aldosterone excretion rate and upright plasma aldosterone rose comparaably in both groups and had returned to pretreatment values within 4 weeks of discontinuing S. In all patients plasma progesterone and 17-hydroxyprogesterone were 2-4 times greater during S therapy than in the pre- or post-treatment periods. In contrast, testosterone, estradiol, FSH, LH, prolactin, 17OH corticosteroids and 17-ketosteroids did not change with S. In 2 menopausal women with undetectable estradiol and high LH and FSH, S-induced changes in progesterone and 17-hydroprogesterone were not significantly different from those of other patients. Patterns of plasma and urinary steroids were not significantly different in patients who developed versus those who did not develop endocrine side-effects.

Significance: We conclude that the increase plasma progesterone and 17-hydroxyprogesterone is probably of adrenal origin. The development of endocrine side-effects can neither be predicted nor accounted for by the changes in plasma concentrations of the hormones measured in this study. The hyperreninemia induced by S is no longer observed 4 weeks after discontinuing therapy with S.

Publications:

1. Stripp, B., Taylor, A. A., Bartter, F. C., Gillette, J. R., Loriaux, D. L., Easley, R. and Menard, R. H.: Effect of spironolactone on sex hormones in man. J. Clin. Endo. and Metab. 41: No. 4, 777-781, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL-01807-02-HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies in Bartter's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Gill, Jr.	Senior Investigator	HE NHLI
OTHER:	A. A. Taylor	Clinical Associate	HE NHLI
	N. Radfar	Staff Fellow	HE NHLI
	R. W. Bowden	Staff Fellow	HE NHLI
	H. R. Keiser	Chief, Sec. of Exper. Therapeutics	HE NHLI
	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any) J. C. Frolich, H. Seyberth and J. A. Oates are from the Clinical Pharmacology Branch, Vanderbilt University, Nashville, Tenn.

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

Urinary prostaglandins are high in patients with Bartter's Syndrome.- treatment with an inhibitor of prostaglandin synthesis decreased urinary prostaglandins by 80 percent, decreased plasma renin activity by 79 percent (supine) and by 76 percent (upright) and decreased urinary aldosterone by 52 percent. Potassium balance was positive and serum K+ rose with treatment in all patients. The resistance to the pressor effects of Angiotensin II and norepinephrine were restored to normal. Renal synthesis of prostaglandins is increased in Bartter's syndrome and prostaglandins mediate hyperreninemia, hyperaldosteronism and the vascular resistance to Angiotensin II and norepinephrine which characterize the syndrome.

681

PHS-6040
(12-75)

Project Description:

Project No.Z01-HL-01807-02-HE

The studies were designed to evaluate the role of prostaglandins in the pathogenesis of Bartter's syndrome. Prostaglandins E_2 and $F_{2\alpha}$ in urine were measured by gas chromatography-mass spectrometry. As these two primary prostaglandins are not filtered at the glomerulus to any appreciable extent, their content in urine reflects their synthesis by the kidney. The major metabolite of prostaglandins E, PGE-M, was also measured in urine and these values for PGE-M are a reflection of total body synthesis of prostaglandins of the E series. After control observations, patients were treated with indomethacin 150 mg per day or ibuprofen 1600 mg per day. The values for urinary prostaglandins before, during andaafter treatment with a prostaglandin synthetase inhibitor and the normal values for women similarly aged are presented in the table below:

	PGE_2 ng/d	$PGF_{2\alpha}$ ng/d	PGE-M µg/d
Control	619 ± 160	873 ± 277	11.04 ± 5.57
Indomethacin	118 ± 21	497 ± 196	2.75 ± 1.30
Post-Control	742 ± 183	968 ± 307	10.91 ± 5.83
Normal Range	76 - 281	422 - 871	3.2 - 14.2

Urinary PGE_2 were high with a mean value more than twice the upper limit of the normal range. PGE-M was normal and suggests that the increase in synthesis of PGE_2 was confined to the kidney. Urinary $PGE_{2\alpha}$ was normal, and indicates that the renal overproduction of prostaglandins was limited to PGE_2. Treatment of the patients with indomethacin, an inhibitor of prostaglandin synthetase decreased urinary PGE_2 by 81 percent. The effects of this inhibition of prostaglandin synthesis on plasma renin activity (PRA) and on aldosterone excretion are shown in the table below.

	PRA ng/ml/hr		AER µg/d
	supine	upright	
Control	12.6 ± 1.8	28.4 ± 3.3	20.8 ± 4.1
Indomethacin	2.6 ± 0.7	6.9 ± 2.8	10.0 ± 1.6
Post-Control	11.9 ± 4.7	26.4 ± 8.5	12.6 ± 3.2

Plasma renin activity (PRA) measured in the supine position and after standing for 3 hours was restored to normal as was urinary aldosterone. The effects of indomethacin or creatinine clearance, the balances of sodium and potassium and serum potassium are shown in the following table:

	Ccr ml/min	Na+ balance mEq	Body Weight kg	K+ balance mEq	Serum K+ mEq/L
Control	95 ± 22	+54	0	+86	2.43 ± .05
Indomethacin	81 ± 20	+395	+2.14	+407	3.22 ± .15
Post-Control	96 ± 20	-137	-0.11	-12	2.59 ± .11

Treatment with indomethacin was associated with a decrease in creatinine clearance which returned to normal when the drug was stopped. Sodium balance was positive and this was reflected by a gain in body weight. The sodium retention was in part the result of a decrease in the filtered load of sodium and may also have been in part the result of an increase in the tubular transport of sodium secondary to probable changes in renal hemodynamics assumed to be the cause of the decrease in creatinine clearance as blood pressure did not change. Potassium balance was positive and led to an increase in serum K+. The decrease in potassium excretion could be attributed in part to a decrease in aldosterone. If the sodium retention were the consequence of an increase in the tubular reabsorption of sodium proximal to the sites in the tubule where potassium secretion occurs, then this could also be a factor in the retention of potassium. These results provide additional evidence for an important role for prostaglandins in the mediation of renin release and suggests that overproduction of prostaglandins by the kidney in patients with Bartter's syndrome is an important intermediary event in the pathogenesis of the hyperreninemia and hyperaldosteronism in the syndrome.

Another feature of the syndrome, resistance to the pressor effects of angiotensin II and of norepinephrine has been hypothesized to be an important feature of the syndrome and the proximal cause. The effects of treatment with indomethacin on this abnormality were studied. The amounts of angiotensin II and norepinephrine required to raise blood pressure 20 mmHg before and after inhibition of prostaglandin synthetase are shown in the table below:

	Control		Indomethacin	
	Angio. II ng/kg/min	NE	Angio. II	NE ng/kg/min
Patients	22.5 ± 5	154 ± 20	8 ± 2	85 ± 20
Normal Range	8-12	60-90		
Mean	10	75		

Thus treatment with indomethacin restored responsiveness to normal and suggests that prostaglandins are an important factor in this resistance. Whereas the nature of this resistance is unexplained, it may be the result of the large amounts of circulating angiotensin II present in the syndrome. In vitro studies indicate that Angiotensin II stimulates the synthesis of prostaglandins by blood vessels and that this generation of prostaglandins is an important determinant of vascular contractility. Thus these studies provide considerable evidence for an important role for prostaglandins in Bartter's syndrome. The question as to what constitutes the proximal event in the pathogenesis of the syndrome, however, must await the results of future studies.

Significance to Biomedical Research and Institute Program: These studies provide information on the important role of prostaglandins in the control of renin release and in determination of blood pressure responsiveness to endogenous pressor substances. As we increase our understanding of Bartter's syndrome, which is characterized by normal blood pressure rather than high blood pressure in the setting of hyperreninemia and hyperaldosteronism, we may gain important insight into the nature of hypertension in addition to elucidation of the syndrome.

Publications:

1. Gill, J. R., Jr., Frolich, J. C., Bowden, R. E., Taylor, A. A., Keiser, H.R., Seyberth, H.W., Oates, J.A. and Bartter, F. C.: Bartter's syndrome: A disorder characterized by high urinary prostaglandins and a dependence of hyperreninemia on prostaglandin synthesis..Amer. J. Med., In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01808 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

"Control of Renin:"
Effect of propranolol on renin release in the dog.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Yun	Staff Fellow	HE NHLI
OTHER:	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Infusion of propranolol in both anesthetized and conscious dogs caused decreases in heart rate, plasma renin activity (PRA), plasma renin concentration (PRC) and plasma aldosterone. There was no change in mean arterial blood pressure, plasma renin substrate concentration, packed cell volume, plasma potassium, or plasma sodium concentration following the infusion of propranolol. In five conscious animals, infusion of propranolol (1 mg/kg followed by 0.47-0.65 mg/kg/hr) reduced heart rate from 117.6 $\pm$ 4.0 beat/min (M $\pm$ SE) during control periods to 73.2 $\pm$ 2.0 beat/min ($P < 0.005$) 90 minutes after the start of infusion. PRA decreased from 9.97 $\pm$ 1.79 ng/ml/hr during control periods to reach 1.50 $\pm$ 0.42 ng/ml/hr ($P < 0.005$) at the end of 90 minutes infusions. The data suggest that propranolol suppresses renin secretion in unstimulated dogs.

PHS-6040
(12-75)

Project Description:

Objectives: It is well established that the sympathetic nervous system plays an important role in the control of renin secretion. Direct renal nerve stimulation and reflexly induced increase in renal nerve activity can cause renin release. It is well known that these stimulatory effects on renin secretion can be blocked by propranolol. It also has been reported that chronic adrenergic receptor blockade with propranolol suppresses plasma renin activity in normal as well as hypertensive patients. It thus seems well established that acute administration of propranolol prevents the renin release induced by experimental adrenergic stimulation and that long term administration of propranolol decreases plasma renin activity in some hypertensive patients. It is, however, still unknown whether acute administration of propranolol would also suppresses plasma renin activity in unstimulated animals. The present series of studies was done to examine this possibility.

Methods Employed: Female mongrel dogs were used in this investigation. In the first series of experiments they were anesthetized with Nembutal (30 mg/kg). One hour after the onset of anesthesia, an arterial blood sample was taken. This was followed by intravenous injection of 5 mg/kg of (d,1) propranolol followed by infusion of 1.12-1.45 mg/kg/hr. Arterial blood samples were then taken every 30 minutes for 90 minutes, and also at 60 minutes after the infusion had been stopped. In the second and third series of experiments, the same protocol was followed, except that the dogs were conscious. In the second series of experiments, propranolol was given at 5 mg/kg followed by 1.32-2.00 mg/kg/hr. In the third series of experiments, propranolol was given at 1 mg/kg followed by 0.47-0.65 mg/kg/hr. Plasma renin activity was determined by radioimmunoassay for angiotensin I and generated per ml of plasma per hour. In five conscious animals, infusion of propranolol (1 mg/kg followed by 0.47-0.65 mg/kg/hr) reduced heart rate from 117.6 $\pm$ 4.0 beat/min (M $\pm$ SE) during control periods to 73.2 $\pm$ 2.0 beat/min ($P<0.005$) 90 minutes after the start of infusion. PRA decreased from 9.97 $\pm$ 1.79 ng/ml/hr during control periods to reach 1.50 $\pm$ 0.42 ng/ml/hr ($P<0.005$) at the end of the 90 minute infusion. The data suggest that propranolol suppresses renin secretion in unstimulated dogs.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01809-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Renal prostaglandins and renal function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Gill, Jr.	Senior Investigator	HE NHLI
OTHER:	R. E. Bowden	Staff Associate	HE NHLI
	J. J. Pisano	Chief, Sec. on Phys. Chem.	HE NHLI
	H. R. Keiser	Chief, Sec. on Exper. Therapeutics	HE NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

Urinary prostaglandin E-like material (iPGE) was measured in the urine of dogs during control observations, during the last forty-minutes of a 2-hour infusion of Ringer's solutions, 10 ml per minute, and during the last forty minutes of a one hour infusion of Ringer's solution and indomethacin, 0.35 mg per minutes. Mean urinary iPGE was 4.69 ± 1.25 ng/min for the left kidney and decreased by 47 percent during Ringer's and by 83 percent during Ringer's plus indomethacin. In another series of studies 2.5 percent dextrose and water, 5 ml/min was infused throughout. Mean urinary iPGE was 3.44 ± 1.23 ng/min for the left kidney and decreased by 48 percent after two hours and by 21 percent after 3 hours. Sodium excretion changed only in the studies with Ringer's. Thus, urinary iPGE exhibits circadian changes and does not appear to be influenced by expansion of extracellular fluid. A decrease in urinary iPGE of greater than 50 percent appears to be necessary for a change in sodium excretion.

Project Description:

Previous studies indicate that infusion of indomethacin into a renal artery of the dog increases the renal excretion of sodium. This effect of indomethacin was observed when the prostaglandin synthetase inhibitor was given alone or when it was given during a natriuresis induced by infusion of Ringer's solution. These results suggest that prostaglandins generated within the kidney stimulate the tubular reabsorption of sodium. Conceivably, the natriuretic effect of expansion of extracellular fluid may depend in part or suppression of renal prostaglandin synthesis. To examine this possibility urinary prostaglandins, which are formed in the kidney, were measured before and during infusion of Ringer's solution and before and during infusion of 2.5 percent dextrose and water in the dog.

Methods Employed: Water diuresis was produced in anesthetized hypophysectomized cortisol treated dogs by infusion of 2.5 percent dextrose. When urine flow was steay control clearance measurements were obtained. In one group of dogs, Ringer's solution, 10 ml/min, was infused for 3 hours with indomethacin 0.35 mg/min given during the last hour. In a second group of dogs, 2.5 percent dextrose solution 5 ml/min was infused for 3 hours. The clearances of inulin and sodium excretion were determined. Measurements of prostaglandin E like material by radioimmunoassay were performed after extraction of urine.

Major Findings: Infusion of Ringer's solution which increased sodium excretion decreased urinary prostaglandin E-like material (iPGE). The addition of indomethacin further increased sodium excretion and decreased iPGE. Infusion of 2.5 percent dextrose and water did not change sodium excretion but was associated with a decrease in urinary iPGE during the first two hours. During the third hour, urinary iPGE returned toward the control value. The results for the left kidney are summarized in the table below:

	$U_{Na}V$ uEq/min	$U_{iPGE}V$ ng/min	C_{In} ml/min	MAP mmHg
Control	11 ± 2	4.69 ± 1.25	32 ± 3	126 ± 3
Ringer's	66 ± 8	2.48 ± 0.5	32 ± 2	134 ± 4
Ringer's and Indomethacin	152 ± 16	0.80 ± 0.25	33 ± 2	143 ± 3
Control	25 ± 6	3.44 ± 1.23	32 ± 3	131 ± 9
2.5% D & W	28 ± 4	1.78 ± 0.58	32 ± 4	130 ± 7
2.5% D & W	36 ± 6	2.72 ± 1.42	33 ± 4	128 ± 8

As the decrease in urinary iPGE during infusion of Ringer's was similar in magnitude to the decrease observed during infusion of 2.5% dextrose had water, this suggests that the change in urinary iPGE is more likely a circadian rhythm than a response to expansion of extraceullular fluid. As sodium excretion increased with Ringer's but not dextrose and water, the change in sodium excretion is probably not a consequence of the change in renal

prostaglandins. The considerable decrease in iPGE with indomethacin below what was observed at a similar time of day in the absecence of indomethacin indicates that indomethacin is an effective inhibitor of prostaglandin synthesis and that this degree of inhibition of prostaglandin synthesis is necessary to affect sodium excretion. Thus the renal synthesis of prostaglandins appear to have in circadian rhythm the importance of this rhythm to sodium excretion is uncertain but may be minor.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01810-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Spironolactone on plasma renin and dog renal histology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Taylor	Clinical Associate	HE NHLI
OTHER:	J. C. Yun	Staff Fellow	HE NHLI
	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI
	J. R. Mitchell	Chief, Sec. of Clin. Pharm.	CP NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

Plasma renin activity (PRA), aldosterone excretion rate (AER) and an index of renal juxtaglomerular cell granulation (JGI) were determined in each of the 6 dogs at monthly intervals during, and 1, 2 and 4 months after the administration of spironolactone, 200 mg per day. PRA and JGI were significantly increased during spironolactone treatment and remained elevated above pre-treatment values as long as 2 months after treatment was stopped. These data suggest that persistent past treatment effects of spironolactone should be considered when evaluating renin-aldosterone or renal histopathology in patients who have taken this drug.

690

PHS-6040
(12-75)

Project Description:

Objectives: Many patients with hypokalemia or hypertension are treated with spironolactone. Thorough diagnostic evaluation of such patients often requires measurement of plasma renin, aldosterone excretion rate, or the examination of renal tissue obtained by biopsy after temporarily stopping drug treatment. It has been reported that the response of plasma renin activity to furosemide and upright posture can remain elevated for as long as 36 weeks after stopping spironolactone therapy in some patients with low renin hypertension (Lowder and Liddle, NEJM 291: 1243, 1974). Post-treatment effects of spironolactone on renal histopathology have not been evaluated.

The purpose of the present study was to evaluate plasma renin activity, aldosterone excretion rate, and renal histopathology in dogs before, during, and after administration of spironolactone.

Methods Employed: 1) Dogs were housed in metabolic cages, fed a diet of known sodium content for 1 week per month, and urine collected daily. 2) After one week of fixed dietary sodium intake, blood was drawn for the measurment of plasma renin activity (measured by radioimmunoassay of generated angiotensin I) sodium, potassium, blood urea nitrogen, creatinine, aldosterone and cortisol (measured by radioimmunoassay). On an aliquot of each daily urine sample were measured sodium, potassium (by flame photometry) and aldosterone (by radioimmunoassay). 3) The dog was then anesthetized with pentobarbital and a small piece of one kidney was removed by retroperitoneal approach using aseptic techniques. 4) For the next 3 weeks each dog was allowed ad libitum food and water and was given a single daily dose of spironolactone, 200 mg. The metabolic balance study and open renal biopsy was then repeated monthly during 3 months of treatment and 1, 2, and 4 months after spironolactone treatment had been stopped. 5) Each piece of renal tissue was divided; one-half was processed and stained with Bowie's stain, the other one-half stained with hematoxylin-eosin. The identification of each slide was masked; an estimation of JG hyperplasia was made on the Hematoxylin-eosin sections and an index of JG cell granularity determined on the Bowie stained sections by a previously described method (Hartroft in Endo. Path., Bloodworth, ed., Williams and Wilkins, 1968, p. 641).

Major Findings: Plasma renin activity (PRA), aldosterone excretion rate (AER), and the renal index of juxtaglomerular cell granulation (JGI) were increased above pretreatment values during the 3 months of spironolactone treatment. AER had returned to pretreatment values one month after stopping spironolactone while PRA and JGI remained elevated for 2 months after spironolactone was discontinued.

Significance: These persistent post-treatment effects of spironolactone in the dog suggest that similar effects should be considered in any patient who has been treated with spironolactone in whom the renin-angiotensin-aldosterone system or renal histopathology is being evaluated.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01812-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Inpatient Hypertensive Diagnostic Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Taylor	Clinical Associate	HE NHLI
OTHER:	J.R. Mitchell	Chief, Sec. on Clin. Pharm.	CP NHLI
	M. Ziegler	Pharmacologist	LCS NIMH
	W.R. Snodgrass	Guest Worker	CP NHLI
	R.A. Rosen	Clinical Associate	KE NHLI
	J.R. Gill, Jr.	Senior Investigator	HE NHLI
	R.J. McMurtry	Clinical Associate	CP NHLI
	F.C. Bartter	Chief, Hyper.-Endo. Br.	HE NHLI
	C.S. Delea	Administrative Assistant	HE NHLI
	J.W. Cox	Biochemist (Supervisor)	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Current methods for classifying hypertensive patients as having high, normal, or low renin in order to identify correctable forms of secondary hypertension are not useful because of a high incidence of both false positives and false negatives. This protocol evaluates the ability of plasma renin following suppression with saline infusion and stimulation with three 40-mg doses of furosemide to accurately predict those hypertensive patients with correctable forms of hypertension. The role of the adrenergic nervous system, the activity of which is assessed by measurement of plasma norepinephrine, in mediating renin release is also being assessed. These studies are in progress in both normotensive and hypertensive subjects; the data are insufficient to reach any final conclusion.

PHS-6040
(12-75)

Project Description:

Objectives: Using a standard protocol (JO2.47H75) we have now evaluated 45 normotensive subjects for comparison with over 150 hypertensive outpatients Analysis of these data indicate that the current evaluation of hypertensive patients often fails to distinguish low from normal renin hypertensives (40% false positives) and usually fails to distinguish high from normal renin hypertensives (70% false negatives).

At present the most definitive way to demonstrate low renin status is by maintaining patients on a 9 mEq sodium diet until they attain sodium balance and then measure plasma renin activity, which will be elevated in normal renin hypertensives. This procedure requires 5-8 days of hospitalization, is expensive, and has poor patient compliance (many patients cannot be absent from work for this interval; others cheat on the low sodium diet). Recent studies (Carey, R.M., Arch. Int. Med. 130: 849, 1972) suggest that the stress to the renin system of a 9 mEq sodium diet for 5-8 days can be duplicated by one day of a low sodium diet plus administration of 120 mg of the diuretic, furosemide, in 3 divided 40 mg doses.

The current plasma renin screening tests are even less sensitive in distinguishing high renin hypertension than they are for low renin because they contain many false negatives, i.e., they classify as normal renin many patients with high renin hypertension (range 40-100% false negatives from various patient series). Thus, patients with renal hypertension often are not studied with more definitive procedures (renal arteriography, renal vein renins) unless other tests are abnormal (e.g., intravenous pyelogram). Recently, Weinberger and Grim (unpublished results) have shown that a more discriminating test for high renin hypertension may be the response of plasma renin to a fourhour intravenous infusion of normal saline (30 ml/kg).

The sympathetic nervous system has long been implicated as having an important pathophysiologic role in the development of maintainance of some types of essential hypertension. Of the many biochemical measures of this system, plasma norepinephrine appears to be the most sensitive in reflecting changes in the sympathetic nervous system in response to acute stimuli such as changes in posture, or exercise. Recent studies indicate that changes in norepinephrine excretion rates may parallel responses of renin to adrenergic stimuli (Esler, M.D. et al., Amer. J. Cardiol. 32: 643, 1973.

Responsiveness of renin and the adrenergic nervous system to a stimulus such as upright posture diminishes with age. Consequently, it is important to carefully select agematched normotensive controls for hypertensive patients in whom the response of renin, aldosterone, and plasma norepinephrine to various stimuli is being measured.

Methods: Normotensive subjects from whom informed consent has been obtained can elect to complete their initial evaluation during one 5hour visit in the outpatient clinic or during a oneday admission to the NIH Clinical Center. This initial evaluation will include a history and physical exam, routine blood chemistries, complete blood count, urinalysis, electrocardiogram, and chest xray. All subsequent studies will be performed in the hospital and will be divided into two separate parts.

Part 1. Evaluation of saline infusion and furosemide administration on plasma renin, aldosterone, and norepinephrine (2 1/2 day hospitalization).

Hospital Day 1: After insertion of an indwelling needle with heparin lock in an arm vein, supine and then 5 minute upright blood samples will be obtained for plasma norepinephrine determinations. After 3 hours of standing, blood for renin, aldosterone, and cortisol is obtained. After one hour in the supine position, normal saline (30 ml/kg body weight) will be infused at a constant rate over a 4-hour interval. Blood pressure will be monitored every 5 minutes during the infusion with a Roche arteriosonde blood pressure recording device. Blood samples will be obtained just before and at the end of the infusion. After terminating the saline infusion, blood will be obtained supine and after 5 minutes of upright posture for norepinephrine. Urine will be collected throughout the day.

Hospital Day 2: A 9 mEq sodium diet will be provided. Furosemide, 40-mg tablets, will be administered at 10 a.m., 2 p.m., and 6 p.m. Blood pressure will be monitored every four hours and urine collected throughout the day to measure sodium and potassium excretion.

Hospital Day 3: After 2 hours of upright posture, blood will be collected for electrolytes, renin, aldosterone, and cortisol. Then an indwelling needle with heparin lock will be inserted into a forearm vein; supine and 5 minutes upright blood samples will be obtained 30 minutes after the needle has been inserted.

Part 2: Effects of a low-sodium diet on renin, aldosterone and norepinephrine response to upright posture. All subjects who have successfully completed part 1 of the study and who have been discharged to eat an ad libitum sodium diet at home for at least two weeks will be asked to participate in part 2 of the study. Subjects may participate in part 1 without participating in part 2 but will not be allowed to complete part 2 without first having successfully completed part 1.

Hospital Days 1 through 5 or 6: Subjects will be admitted to the Clinical Center, fed a 9 m Eq per day sodium diet, and will collect urine in 24-hour aliquots for measurement of sodium and potassium. When daily sodium excretion is 10 mEq or less, they will stand for 2 hours at which time blood for electrolytes, renin, aldosterone, and cortisol will be obtained. Then an indwelling needle with heparin lock will be inserted

into a forearm vein and blood obtained supine and then after 5 minutes standing for norepinephrine determinations. The subject will then be discharged.

Comment:

The ability of the one day furosemide to stimulate the effects of a very low sodium intake on the renin, aldosterone and norepinephrine response to upright posture can be evaluated in the same subject using this study design.

Major Findings:

The studies are currently in progress and insufficient data have been obtained to reach any final conclusion.

Significance:

As indicated under objectives.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01813-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of Angiotensin on Aldosterone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.R. Mitchell	Chief, Sec. on Clin.Pharm.	CP NHLI
OTHER:	R.A. Rosen	Clinical Associate	KE NHLI
	A.A. Taylor	Clinical Associate	HE NHLI
	W.R. Snodgrass	Guest Worker	CP NHLI
	F.C. Bartter	Chief, Hyper.→Endo. Br.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with low renin hypertension have low renin and angiotensin II concentrations but aldosterone values which are normal and, therefore, inappropriately high in relation to the suppressed renin. Previous studies have indicated that sodium-retaining steroids other than aldosterone are not involved in the maintenance of hypertension in these patients. Among other explanations is the possibility that the adrenal receptor sensitivity to angiotensin II is increased in patients with low renin hypertension. This protocol evaluates that hypothesis by examining the aldosterone response to infused Angiotensin II in normotensive subjects and in low renin hypertensive patients. Four patients with low renin hypertension and 2 normotensive subjects have been studied but the results are currently insufficient to reach any conclusions.

PHS-6040
(12-75)

Project Description:

Objectives: Patients with low renin hypertension (LRH) have low renin and Angiotensin II (AII) concentrations but normal aldosterone values. The aldosterone is inappropriately high in relation to the suppressed renin and AII, yet a diagnosis of primary aldosteronism is excluded by current criteria because aldosterone excretion rates do not exceed the wide range of normal.

Several investigators have proposed that there is an overproduction of one or more non-aldosterone Na^+-retaining steroids in order to explain the suppressed renin as well as the antihypertensive actions of spironolactone and aminoglutethimide (an inhibitor of adrenal steroidogenesis) in these patients. However, initial studies by our group have failed to document the overproduction of such steroids. In addition, we have noted that while blood pressure is being controlled in these patients by aminoglutethimide, only aldosterone, and not other adrenal steroids, is decreased. Furthermore, Baxter, et al. (Clin. Res. 23: 386A, 1975) have developed a renal receptor assay for Na^+ retaining steroids; with this assay no Na^+-retaining activity other than aldosterone has been detected in the plasma of our low renin hypertensive patients. Finally, an excess of another sodium-retaining steroid should suppress aldosterone as well as renin.

We have thus been led to consider additional hypotheses for the pathogenesis of LRH which are amenable to experimental analysis 1) Hypothesis: adrenal receptor sensitivity to Angiotensin II in patients with low renin hypertension is increased and thus subnormal amounts of renin and angiotensin II produce normal aldosterone secretion rates.

2) Hypothesis: LRH is an early form of Conn's Syndrome in which autonomous aldosterone secretion is suppressed minimally by saline infusion and is stimulated subnormally by angiotensin II.

Methods: Patients will undergo an extensive clinic evaluation including a complete history and physical exam, chest xray, electrocardiogram, intravenous pyelogram, renogram, urinalysis, electrolytes, creatinine clearance and characterization of their reninangiotensin system. Hypertensive patients who show hyporesponsiveness of their plasma renin activity (PRA) to upright posture with respect to their 24-hour $U_{Na}V$ (the Laragh criteria for LRH) (Laragh, J.H. et al., Amer. J. Med. 52: 633, 1972) and do not have renal failure, congestive heart failure, angina pectoris, history of prior myocardial infarction, central nervous system vascular disease, cardiac arrhythmias or malignant hypertension will be admitted to the study. There will be a 5-day hospitalization with the following schedule:

Day 1,2: Patient will be placed on a 109 Na^+, 70 K^+ diet to assure similar Na^+ intakes among the patients, and to permit their blood pressure to fall to a lower resting value. On day 2, the patient will be kept supine and 12 ml blood will be obtained at 0, 60 and 120 minutes for

plasma aldosterone, cortisol, renin and potassium.

Day 3: Angiotensin II will be infused at a fixed dose (2 ng/kg/min) for 1 hour with the patient supine and monitored by blood pressure determinations every 2 minutes and by continous electrocardiogram. This is a subpressor dose of Angiotensin II in most normotensive and hypertensive patients (9,10). Should a rare patient show an excessive rise in blood pressure, the infusion will be stopped and resumed at a lower dose so that blood pressure does not exceed 190 mmHg systolic or 120 mmHg diastolic. Blood will be withdrawn via a venous heparin lock at 0, 30, 45, 60 and 120 minutes for plasma aldosterone, cortisol, renin and potassium determinations.

Day 4: Angiotensin II will be infused in a dosepressor response manner until diastolic blood pressure is raised by 20 mmHg. This dose will then be infused for 1 hour under the above conditions and blood samples (12 ml) will again be obtained at 0, 30, 45, 60 and 120 minutes. No patients blood pressure will be allowed to exceed 190 mmHg systolic or 120 diastolic.

Day 5: The patient will be kept supine and normal saline (30 ml/kg or approximately 2 L.) will be infused at a constant rate over a 4-hour interval. Blood samples (12 ml) will be drawn at 0 and 4 hours for plasma aldosterone, cortisol, renin and potassium determinations. Blood pressure will be measured every 5 minutes, and the infusion discontinued if there are signs or symptoms of fluid overload, or a rise in systolic pressure to 190 or diastolic to 120.

Comments:

Ideally one would like to define the sensitivity of adrenal aldosterone secretion to Angiotensin II over a wide dose range in order to evaluate both hyposensitivity as well as hypersensitivity. This is not possible with Angiotensin II because of its additional dose-limiting pressor action, which is greater in hypertensive than normotensive patients (Distler, A. et al., Europ. J. Clin. Invest. 1: 96, 1970) (Mode of sensitivity: 5vs. 10 ng/kg/min, respectively). Unfortunately, stimulation of aldosterone secretion often requires I.V. doses of Angiotensin II > 3 ng/kg/min) (10). Thus, we have elected to assess hypersensitivity of aldosterone secretion to Angiotensin II be administering the largest fixed dose (2 ng/kg/min) that all hypertensive patients can usually tolerate without excessive blood pressure elevation (Protocol Day 3), and to assess hyposensitivity by administering as large a dose as seems appropriate, i.e., a dose that increases diastolic blood pressure by 20 mmHg (Protocol Day 4).

Major Findings:

Four low-renin hypertensive patients and 2 normotensive subjects have been studied but the results are currently insufficient to reach any final conclusions.

Significance:

The maneuvers may provide better methods of diagnosing the state of low renin hypertension (LRH). We may discover an increased adrenal aldosterone secretion response to Angiotensin II. This could be either a primary disturbance or a compensation for low renin levels because of juxtaglomerular (JGA) hyporesponsiveness to normal beta adrenergic stimuli. More detailed analysis of the defect in the control mechanism would then require further study.

Conversely, we may be able to define relatively large segments of the total population of essential hypertensives as having an early form of Conn's Syndrome. These patients should then have their renin-angiotensin-aldosterone system followed closely in order to establish the natural history of this disorder, and also to be able to detect, at the earliest possible stage, the overt appearance of an adrenal adenoma - a potentially curable form of hypertension.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-01814-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The effect of triamterene in blood pressure, the renin-aldosterone axis and sex steroids.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. Rosen	Clinical Associate	HE NHLI
OTHER:	J. R. Mitchell	Chief, Lab. of Clin. Pharm.	CP NHLI
	A. A. Taylor	Clinical Associate	HE NHLI
	D. E. Rollins	Research Associate	CP NHLI
	W. R. Snodgrass	Guest Worker	CP NHLI
	R. J. McMurtry	Clinical Associate	CP NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of triamterene to control blood pressure, alter the renin-aldosterone axis and cause clinical and biochemical side effects is being assessed. In addition, because of the many undesirable side effects of spironolactone, triamterene is being evaluated as a suitable alternative potassium-sparing diuretic in the treatment of hypertension. Triamterene has been shown to be an effective antihypertensive agent in most patients with fewer side-effects, to date, then spironolactone. Further analysis needs to be done to correlate antihypertensive effect with renin status, and also to determine the mechanisms of fall in creatinine clearance and of polydipsia.

701

PHS-6040
(12-75)

Project Description:

Objectives: The purpose of this study is to assess the ability of triamterene to control blood pressure, to alter the renin-aldosterone axis, and to cause clinical and biochemical side effects. In addition, because the only other major potassium-sparing diuretic, spironolactone, has many undesirable side effects, triamterene will be reviewed in regard to being a suitable alternative to spironolactone in the treatment of hypertension.

Methods: Blood pressure was measured while patients were hospitalized and when seen in the outpatient clinic. Patients also took their own blood pressure at home and considerable weight was given to their home blood pressure records in evaluating their treatment. Routine blood and urine tests were done by the NIH Clinical Laboratories and hormone assays (renin, aldosterone, cortisol and sex steroids) were performed by Hazleton Laboratories.

Major Findings: Triamterene is a significant antihypertensive agent and adequate lowering of blood pressure was noted in 12 of the 18 hypertensive patients treated with this drug alone. Several patients noted gastrointestinal upset initially which tended to abate with time, and almost every patient noted the development of significant thirst. There were four patients whose creatinine clearance fell by 30% in spite of apparently normal urine volumes significant weight loss. Serum potassium rose from pretreatment hypokalemia (in those who have previously taken diuretics) to normokalemia, while normokalemia was maintained in the remainder with one exception. This patient, who had a significant fall in creatinine clearance, developed mild hyperkalemia. There was a significant and gradual rise in plasma renin activity in all patients with continued therapy. There was no change in sex steroid levels. Two patients developed a drug related rash.

Significance: Triamterene has been shown to be an effective antihypertensive agent in most patients. Further analysis needs to be done to correlate antihypertensive effect with renin states, and also to determine the mechanism of the fall in creatinine clearance, and to determine whether the polydipsia is secondary to a CNS or renal action of the drug.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01815-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Role of prostaglandins in the control of renin secretion.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Yun	Staff Fellow	HE NHLI
OTHER:	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.7	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

The role of prostaglandins in the control of renin secretion was examined in dogs maintained for one to two weeks on a diet containing 4 mEq Na+/day. Infusion of indomethacin into the left renal artery (0.5 mg/kg followed by 388 ug/min) caused significant increases in mean arterial blood pressure, and significant decreases in urine flow rate, renal blood, and renin secretion rate. Subsequent infusion of prostaglandin E_2 (PGE_2) at 4.12 ug/min into the left renal artery caused significant increases in urine flow rate, renal blood flow, and renin secretion rate. The data suggest that PGE_2 plays a role in the control of renin secretion.

PHS-6040
(12-75)

Project Description:

Prostaglandins have been found in a wide variety of animal tissues and appear to possess some biological activites in the preparations in which they have been studied. It has been reported that synthesis of prostaglandins of the E and F series takes place in the kidney and that those of the E series are released following many types of stimuli. Although much work has been done to examine the role of prostaglandins in renal function, relatively little has been done to examine their possible role in the control of renin secretion. The purpose of this investigation was to examine this possibility. It appeared that if prostaglandins play a role in the control of renin secretion, infusion of a prostaglandin synthetase inhibitor into the kidney should cause a decrease in renin release. Furthermore, infusion of prostaglandins into the kidney should lead to an increase in renin secretion.

Mongrels dogs maintained for one to two weeks on a diet containing 4 mEq of sodium each day were used in the experiments. They were anesthetized with nembutal and ventilated with a respirator. Plasma renin activity was determined by radioimmunoassay for angiotensin I.

Infusion of indomethacin into the left renal artery (0.5 mg/kg followed by 388 ug/min) caused left renal vein plasma renin activity to decrease from 82.88 $\pm$ 12.77 ng/ml/hr (M $\pm$ S.E.) during control periods to 42.04 $\pm$ 5.40 ng/ml/hr ($P < 0.01$) by 80 minutes after the start of the infusion of indomethacin. Subsequent infusion of prostaglandin E_2 (4.12 ug/min) into the left renal artery for 60 minutes caused PRA to increase to 92.88 $\pm$ 5.35 ng/ml/hr ($P < 0.005$). The data suggest that PGE_2 plays a role in the control of renin secretion.

Publications:

1. Johnson, S.A., Webber, A.J., Wojik, J.D., and Yun, J.: The thrombogenic elements in arterioles. In: Johnson, S.A. and Guest, M.D. (Eds.): Dynamics of Thrombus Formation and Dissolution, Philadelphia, Pa., Lippincott Co., 1969, pp. 72-94.

2. Yun, J.C.H., Delea, C.S., Bartter, F.C., and Kelly, G.: Increase in renin release after sinoaortic denervation and cervical vagotomy. Amer. J. Physiol., In press, 1976.

3. Yun, J.C.H., and Kenney, R.A.: Preparation of cat kidney tissue for ultrastructural studies. J. Electronmicroscopy, In press, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01821-02 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Nephrogenous cyclic AMP as a parathyroid function test.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. E. Broadus	Staff Associate	HE NHLI
OTHER:	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any)

J. E. Mahaffey and R. M. Neer, Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts.

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of these studies has been to demonstrate that nephrogenous cAMP provides a sensitive and specific in vivo bioassay of parathyroid function. In a group of 58 patients with primary hyperparathyroidism, iPTH was elevated in 42 (71%) and nephrogenous cAMP in 52 (90%) of patients. Based on the physiology of cAMP clearance and the findings of this study, a parametric expression for urinary cAMP (nmol/min/100 ml GFR) was developed. This simple analysis and expression revealed abnormal values in 90% of hyperparathyroid patients. Patients with primary hyperparathyroidism and episodic hypercalcemia constitute a common and difficult diagnostic problem; the abnormal parathyroid function in these patients may be easily defined by lack of normal suppression of nephrogenous cAMP in response to calcium infusion. Nephrogenous cAMP has also been used to help develop diagnostic criteria in the ill-defined subtypes of idiopathic hypercalciuria. Patients with "renal leak" hypercalciuria display high fasting calcium excretion, secondary hyperparathyroidism and normal parathyroid suppressibility. Patients with "absorptive" hypercalciuria display normal fasting calcium excretion and suppression of nephrogenous cyclic AMP in response to oral calcium.

PHS-6040
(12-75)

Project Description:

The main objective of the study has been to demonstrate that nephrogenous cyclic AMP offers a valid index of parathyroid function in a variety of clinical and investigative situations.

The major clinical areas studied thus far include: 1) the diagnostic utility of the cyclic AMP analyses in a large group of patients with primary hyperparathyroidism, 2) development of diagnostic criteria for patients with primary hyperparathyroidism and episodic hypercalcemia, 3) development of diagnostic criteria for defining subtypes of idiopathic hypercalciuria.

It should be noted that the validation of the clinical utility of the cyclic AMP measurements comes at a particularly apt time, in that the limitations of the methodology for determining serum immunoreactive parathyroid hormone (iPTH) are being increasingly recognized. These limitations are due to the heterologous assay systems in current use, the heterogeneity of circulating forms of the peptide and the lack of a standardized methodology.

Methods Employed: The methods were adequately detailed in the 1974-75 Project Report.

Major Findings: Results $\pm$ SD in control subjects and in patients with parathyroid disease were as follows:

Patients (n)	Serum Ca (mg/dl)	iPTH (uleq/ml)	CR (CcAMP/Ccr)	Urinary cAMP (nmols/min/100 ml Ccr)
HypoPT (10)	7.9 $\pm$ 0.8	32 $\pm$ 6#	1.23 $\pm$ 0.09	2.16 $\pm$ 0.38
Control (55)	9.4 $\pm$ 0.4	59 $\pm$ 19	2.01 $\pm$ 0.43	3.19 $\pm$ 0.69
HyperPT (58)	11.1 $\pm$ 0.9	145 $\pm$ 100	3.62 $\pm$ 0.88	6.33 $\pm$ 2.07

#undetectable in 4 patients

In the control group, 95% of values were: iPTH $\leq$ 90 uleq/ml, CR $\leq$ 2.72, and urinary cAMP $<$ 4.31 nmols/min/100 ml Ccr. The CR was elevated in 90%, urinary cAMP in 90%, and iPTH in 71% of the hyperPT patients, and similar data were obtained in subgroups of 1) 26 patients with mild or episodic hypercalcemia and 2) 10 patients with moderate azotemia (mean Ccr 43 ml/min). Urinary cAMP was low and the CR near unity (mean 1.18, range 0.78-1.36) in non-azotemic and azotemic patients with hypoPT (n=10) and non-PT hypercalcemia (n=4); both cAMP analyses were elevated in ectopic hyperPT (n=3). Values for iPTH were less readily interpreted in these groups of patients.

An important aspect of this series was the development of a parametric, physiologically rational expression of urinary cyclic (nmol/min/100 ml GFR), which avoids serious interpretive problems internet in other expressions and which validly reflects parathyroid activity over a wide (greater than 10-fold) range of renal function. This simple analysis and expression should consititute a widely available test for biochemical hyperparathyroidism.

Patients with primary hyperparathyroidism and episodic hypercalcemia are regarded as one of the most difficult differential diagnostic problems in calcium metabolism. The recent description of renal hypercalciuria with secondary hyperparathyroidism (see below) has further clouded the diagnostic picture. Based on the current literature, iPTH results in response to calcium infusions in such patients are essentially impossible to interpret (our experience has confirmed these findings). The analysis of nephrogenous cyclic AMP during such infusions, however, clearly defines the abnormal parathyroid function in these patients. In essence, failure to suppress parathyroid function (as defined by nephrogenous cyclic AMP) by more than 30% identifies the primary parathyroid abnormality, and operative proof has been obtained in 5 of 6 such patients.

There continues to be much debate and controversy concerning the pathogenesis of idiopathic hypercalciuria. Evidence is mounting that there are two subtypes of this disorder, although diagnostic criteria remain somewhat ill-defined. Current studies in our laboratory suggest the following preliminary criteria:

Absorptive hypercalciuria:
1. increased calcium absorption
2. normal fasting calcium excretion
3. normal basal parathyroid function
4. often hypercalcemia after oral doses of calcium
5. suppression of nephrogenous cyclic AMP by $\geq$ 50% after oral doses of calcium

"Renal Leak" Hypercalciuria:
1. increase calcium absorption (secondary)
2. elevated fasting calcium excretion
3. elevated basal parathyroid function
4. low normal (or frankly low) serum calcium with only slight change after oral doses of calcium.
5. only slight suppression of parathyroid function after oral doses of calcium.
6. normal parathyroid suppressibility to calcium infusion.

Based on the various findings noted above, it is our feeling that we can reach a definitive diagnosis in virtually 100% of patients presenting with recurrent calcium stone formation and hypercalciuria and properly sort the patients into the categories of 1) primary hyperparathyroidism with episodic hypercalcemia, 2) absorptive hypercalciuria or 3) renal hypercalciuria.

Publications: One manuscript has been submitted for publication and three additional manuscripts are in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01822-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of aminoglycoside antibiotics on urinary calcium clearance.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Licata	Staff Fellow	HE NHLI
	R. J. McMurtry	Clinical Associate	CP NHLI
OTHER:	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any) Section on Clinical Pharmacology, NHLI, NIH

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The acute effects of gentamycin and kanamycin on urinary electrolytes were tested in human volunteers and rats. The data from laboratory animal experiments indicated large doses of aminoglycosides produced a specific increase in calcium excretion. In human volunteers this acute effect was not noted in the group or a whole.

PHS-6040
(12-75)

Project Description:

Objectives: Renal magnesium wasting with concomitant gentamicin induced suppresion of parathyroid hormone secretion has recently been implicated in the development of hypomagnesemic hypocalcemia in patients receiving prolonged gentamicin therapy. Hypocalcemia and hypomagnesmia have also been associated with the prolonged use of capreomycin, viomycin and multi-drug antitubercular regimens containing gentamicin.

We report here the effects of acute administration of aminoglycoside antibiotics in urinary electrolyte excretion in animals and in human volunteers.

Methods: Human studies- Metabolic balance studies were performed in seven young adults (five females and two males) age 20 to 23. Subjects received a daily diet containing (600) mg calcium, (100 mg) phosphorus, (69 mEq) sodium, and magnesium (100-150 mg). Intramuscular injections of either kanamycin (Kantrex, Bristol Laboratories) 7.5 mg/kg b.i.d. or gentamycin (Garamycin, Schering Corporation) were given to each volunteer for a 24 hour period. Urine was collected every four hours for two days prior to, during, and for two days after, drug administration. Urine was analyzed for Ca, P, Mg, Na and K by atomic absorption spectrometry. Serum Ca, P, Mg, Na, K, Cl, Co_2, BUN, creatinine, albumin, total protein, glucose, uric acid, SGOT, SGPT, LDH, alkaline phosphatase were measured by autotechicon before, and 24 hours after the study.

Animal studies- Male spraque-Dawley rats 200-210 g were housed in glass metabolic cages for four hours following the administration, intraperitonealy of aminoglycoside (200 mg/kg) or normal saline. At the end of this period the animals were sacrificed and the aspirated urinary bladder contents were combined with collected urine and a de-ionized water rinse of the collecting apparatus. Urinary electrolytes and protein determinations were performed by Bionetics Medical Laboratories, Kensington, Maryland.

Major Findings: In rats, the acute administration of gentamycin or kanamycin aminoglycoside produced an increase in urinary calcium excretion within a four hour period without affecting the excretion of other electrolytes. Urinary protein loss was also unaltered during this time. Although a diverse group of antibiotics and nephrotoxins were evaluated in a similar manner (Unpublished data, Laboratory of Chemical Pharmacology) only the aminoglycoside antibiotics were associated with acute hypercalciuria. As a group, the human, volunteers, did not show altered excretion of divalent cations when given acute doses of these same antiobitics. However, one subject did show a transient increase in magnesium excretion following the initial dose of gentamycin but failed to sustain this level of excretion after subsequent injections. Urinary sodium and potassium and serum chemical values were also unaltered during this study. Our data do not support acute effects of these aminoglycosides on urinary divalent cations excretion in human volunteers. Although the animal data show an acute effect, the administered doses were large compared to the therapeutic levels used in

human subjects. This observation may mean that the reported cases of hypocalcemia and hypomagnesemia could have results from the effect of cummulative doses of the gycoside on renal function directly, on parathyroid gland metabolism or both.

Publications: None.

Table I: Urinary electrolytes excretion in adult, male spraque-Dawley rats receiving a single intraperitoneal injection of kanamycin, 200 mg/kg, or gentamycin, 200 mg/kg.

Urinary excretion treatment	Na+(mEq)	K+(mEq)	Ca++(mg)	Mg++(mg)	Protein (m)
Control					
1.0 cc/kg i.p. NS	0.291[a]	0.405	0.17	0.27	1.89
	±0.79	±1.04	.07	.05	±0.31
Gentamycin	0.102	0.297	1.07[b,c]	0.24	1.68
	0.90	0.80	0.19	0.06	0.08
Kanamycin	0.292	0.478	1.04[b,c]	0.29	1.02
	0.72	0.43	0.64	0.02	0.97

[a]Value represents the four hour urinary excretion following injection as described in methods.

[b]Data represents mean of 3 animals ± standard deviation.

[c]Significant at level P 0.05.

Table II: Urinary calcium and magnesium excretion in individuals receiving intramuscular kanamycin[a], 7.5 mg/kg, or gentamycin[a], 60-80 mg.

Aminoglycosides	mg/24 hrs. divalent cation	Day 1	2	3	4	5
Kanamycin[a]	Ca++b	138 ±6	160 ±50	168 ±34	128 +44	132 ±28
	Mg++c	124 ±31	128 ±19	128 ±19	107 ±16	123 ±49
Gentamycin[a]	Ca++c	231 ±23	210 ±31	200 ±31	170 ±14	174 ±10
	Mg++c	87 ±24	81 ±24	110 ±64	85 ±25	82 ±23

[a]Aminoglycoside administered over a 24 hour period on Day 3.

[b]Data represents mean of 4 individuals ± standard error.

[c]Data represents mean of 3 individuals ± standard error.

Table III: Acute effect of gentamycin on divalent cation excretion (mg/3 hrs).

Cation	Day 2					Day 3					Day 4				
	Period of Collection														
	1	2	3	4	5+6	1*	2*	3*	4	5+6	1	2	3	4	5+6
	21	15	15	23	29	77	19	21	14	50	24	18	24	18	27
Ca++	46	35	33	61	48	40	44	48	32	59	38	32	41	31	26

*Gentamycin was given at periods, 1, 2 and 3.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01824-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Prostaglandin E_1 - A possible regulator of parathyroid hormone secretion in vitro.
Chemical regulators of parathyroid gland secretion.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Licata	Staff Fellow	HE NHLI
	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any)
William Y. Au, Department of Pharmacology, University of Rochester, School of Medicine, Rochester, NY.

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

SUMMARY OF WORK (200 words or less - underline keywords)

Prostaglandin E_1 was studied for its effects on protein and glycoprotein metabolism in rat parathyroid glands. Concentration of PGE_1 (100 µg/ml) produced significant decreases in the incorporation of radioactive uridine and thymidine into RNA and DNA respectively. A similar dose failed to alter the incorporation of radioactive fucose or amino acids into cellular glycoprotein. or protein. However, this dose and lower doses (0.0001 µg/ml) did inhibit secretion of immunological parathyroid hormone. Cortisol and vitamin A appeared to block this effect of PGE (0.01 µg/ml) on secretion of radioactively labeled parathyroid hormone and of the specific glycoprotein.

PHS-6040
(12-75)

Project No. Z01-HL-01824-02 HE

Project Description:

Objectives: Present evidence has shown that the secreting parathyroid hormone is regulated by many factors in addition to calcium. Prostaglandins have been implicated as regulators in many hormone-producing organs. The possibility of prostaglandin regulation of parathyroid gland metabolism was investigated *in vitro*.

Methods Employed: Rat parathyroid glands were studied in an *in vitro* system. Radioactive precursors of protein, glycoprotein, DNA and RNA were used to investigate metabolism of these glands with and without prostaglandin E_1.

Major Findings: Rat parathyroid glands take up and incorporate radioactive amino acid (circles) and L-fucose (circles) into cellular proteins and glycoproteins (Figure 1). Part of these radioactive proteins and glycoproteins are secreted into the culture medium and can be resolved into three major peaks of radioactivity (Figure 2). The smallest peak (peak 3) eluting before the free isotopes contains radioactively labeled protein and glycoprotein and immunoreative parathyroid hormone. There was a close correlation between the amount of secreted parathyroid hormone and the amount of secreted labeled protein and glycoprotein in this peak 3 (Figure 3). At high doses (100 ug/ml) PGE_1 inhibited incorporation of uridine and thymidine into RNA and DNA respectively, but failed to do so at lower concentrations. No concentration of PGE_1 employed altered incorporation of labeled precursors into cellular protein or glycoprotein. PGE_1 did block release of parathyroid hormone, but not in a log dose response. PGE_1 at 0.01 ug/ml specifically inhibited the release of protein and glycoprotein material in peak 3 (Figure 4) by 50% and also the release of immunological PTH. This inhibition of peak 3 release was blocked by vitamin A (1.0 ug/ml) and cortisol (12 ug/ml) (Figure 5).

Significance: Hypercalcemia is a relatively common finding on routine laboratory tests. What factors control serum calcium remain to be elucidated. This *in vitro* technique serves as a useful model to test the validity of various assumptions about regulators of parathyroid gland metabolism. The finding that prostaglandin E can specifically blcok secretion of PTH implies that another class of biological agents may be an important factor in parathyroid gland function. Further studies are warranted to substantiate whether *in vivo* these effects of PGE_1 are found.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01825-02 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less) Evaluation of Sodium Cellulose Phosphate:" Effects of dietary protein on urinary calcium loss in normals and patients with nephrolithiasis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Licata	Staff Fellow	HE NHLI
OTHER:	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI
	E. Bou	Dietician	CC NUTR

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Acute alterations of urinary and serum calcium in normal volunteers was analyzed following low (8 gm) and high protein (24 gms) load with low (350 mg) and high (1000 mg) oral calcium supplements. Two hours following an acute oral load of 350 mg or 1000 mg of calcium, serum calcium rose to an average of 0.23 mg/dl and 0.88 mg/dl, respectively. Urinary calcium increased one to two hours after the load and averaged 0.104 mg/mg Cl and 0.035 mg/mg Cr following the low and high calcium loads. With a protein load, urinary calcium rose to greater degree when protein was added to the low calcium load than when added to the high calcium load. There were inconsistent changes in titratable acid or ammonia production noted. In some patients with nephrolithiasis, it was possible to control the urinary loss of calcium by altering dietary protein with Ca intake constant at 400-500 mg/day.

716

Project Description: Project No. Z01-HL-01825-02 HE

Objective: To analyze the effect of acute protein loads on the excreting urinary calcium in normals and to determine the effect of dietary protein on calcium loss in patients with nephrolithiasis.

Methods: Metabolic balance technique were used to study the chronic effect of protein on urinary loss of calcium in the patient with nephrolithiiasis while maintained on a diet of 400-500 mg calcium. Normal volunteers were given 350 and 1000 mg calcium loads, with and without loads of protein (8 gm and 24 gms). Urine was analyzed hourly over four hours for Ca, P, Na, K, Mg, Cl, titratable acid, and ammonia.

Findings: Preliminary results (Table I) showed that the acute calcium loads alone produced an increase in urinary calcium during the second hour after an oral load. In the presence of added protein, these calcium loads produced a greater and sustained increase in urinary calcium loss. Further data concerning the effects of urinary phosphorus, magnesiu, sodium, potassium, chloride, nitrogen, and titratable acid and ammonia are being computed. In patients with nephrolithiasis, a low protein diet lowered the excretion of calcium while the calcium was maintained at 400-500 mg/day (Table II). Increasing only dietary protein produced an increase in urinary calcium excretion which could be corrected by sodium cellulose phosphate (SCP) in two of the three patients studied. These two patients who previously showed good therapeutic responses to SCP a chelatn of intestinal calcium were known hyperabsorbers of calcium. The effects of SCP in counteracting the protein induced loss of calcium in the urine suggest that protein may increase calcium absorption in some patients. In the other patient however who showed no benefit from SCP some other mechanism of protein action must be involved.

Significance: In our previous work, we have noted changes in calcium absorption, changes in urinary electrolyte excretion in acid base status, and calcium balance. These present studies attempt to answer whether protein can produce acute changes in calcium loss similar to those seen during chronic periods. That dietary protein alone can alter urinary calcium loss is important for patients with nephrolithiasis. Reduction in dietary calcium alone is advised in such patients. Our studies show that this is not enough. Protein also needs to be restricted in order to control urinary calcium. In addition, the use of sodium cellulose phosphate may not be therapeutically effective in the presence of high protein in the diet in some people.

Publications: None.

Table I
The effect of calcium and protein
loads on urinary calcium excretion

Calcium (mg)	Protein (gm)	Urine Calcium (mg/Cr) Time (hrs post load) 1	2	3	4
350	0	0.09*	0.19	0.16	0.12
	8	0.10	0.30	0.26	0.20
	25	0.10	0.20	0.28	0.30
1050	0	0.09	0.13	0.20	
	8	0.15	0.27	0.33	0.35
	25	0.11	0.22	0.24	0.28

*Mean values 3 from volunteers.

Table II
The effect of dietary protein on urinary
Calcium in patients with nephrolithiasis

Patient	Urine Calcium (mg/d) Low Protein	High Protein	High Protein + SCP*	High Protein
1	188	350	325	———
2	242	308	87	262
3	235	329	142	412

*SCP = sodium cellulose phosphate

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01826-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The excretion of phosphoethanolamine in various disease states

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Licata	Staff Fellow	HE NHLI
OTHER:	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI
	N. Radfar	Staff Fellow	HE NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

These studies show that phosphoethanolamine excretion can no longer be considered pathognomonic for the disease hypophosphatasia. Variation in normal physiology and diet significantly alter daily excretion of the substance. In addition, the increased amounts of PEA excreted in various bone diseases, hypertension, and different endocrine disorders implies that a fundamental process common to many diseases results in the increased loss of PEA.

719

PHS-6040
(12-75)

Project Description:

Objectives: The presence of phosphoethanolamine (PEA) in the urine has been used as a marker for the metabolic bone disease hypophosphatasia. This disease is characterized by low levels of serum alkaline phosphatase, premature shedding of deciduous teeth, a "rickets-like" bone histology, and spontaneous fractures. The disease can manifest itself during infancy, adolescence, or adulthood but the more severe homozygous form, which displays all the above characteristics, appears earlier in life. The amount of urinary PEA excreted daily has been used as a screening procedure to identify heterozygous carriers of the trait because these individuals excrete only one-fifth to one-tenth as much as the homozygotes but three to five times more than normals. In our study we have investigated the secretion of PEA in patients with osseous (excluding hypophosphatasia) and non-osseous diseases, and the effect of diet on this excretory pattern.

Methods Employed: Normal volunteers and patients with bone disease, hypertension, nephrolithiasis and various endocrine disorders supplied 24-hour urine collections for phosphoethanolamine and creatinine analyses. In certain cases, subjects were studied while they were in the metabolic unit and were given diets containing constant sodium (69 mEq), potassium, phosphorus (1000 mg/day) and calcium (700-1200 mg/day) and different meatless protein-loads (0.5 gm and 2.0 gm protein per kg). Serum was analyzed by autotechnicon for calcium, phosphorus, alkaline phosphatase, and creatinine. Urinary electrolytes were determined by flame photometry and atomic absorption spectrophotometry. Phosphoethanolamine was measured by column chromatography using the Moore-Stein technique.

The patients with clinically apparent bone disease included post-menopausal osteoporosis, idiopathic osteoporosis, osteitis fibrosa cystica, rheumatoid arthritis "disappearing bone disease". The group of hypertensive patients were "essential hypertensives" and were taking various medications for control of their disease. The patients with nephrolithiasis were being treated with hydrochlorothiazide and/or sodium cellulose phosphate and dietary restriction of calcium.

Major Findings: PEA excretion in normals: The absolute amount of PEA excreted daily by the normal individuals varied widely as a function of age. The younger group however (ages < 15) excreted more PEA per gram creatinine than anyone of the older groups. In addition to the individual variability of daily PEA excretion, individuals showed a circadian variability in the daily excretion of PEA. Analyses of PEA in the 4-hour urine specimens (beginning at 8 a.m. on day 1) over a 4-day period showed a marked degree of change in PEA excretion during a 24-hour period. In general, there was a tendency for PEA excretion to rise during the late evening and early morning hours. This trend persisted even if PEA excretion was corrected for creatinine (data not shown).

Influences on dietary protein on PEA excretion. When a similar circadian

analysis of PEA excretion was made during periods of two different dietary protein intakes, a striking increase in the amount of PEA excreted was noted in a normal subject and in two patients with osteoporosis. The added dietary protein accentuated the excretion of the late evening-early morning peaks of PEA and produced on overall increase in daily PEA excretion without significant changes in creatinine excretion. Normals and patients with bone disease (with the exception of one patient) significantly increased their excretion of PEA during the high protein diet 43-44%.

PEA excretion in disease states: The absolute amount of PEA excreted daily by patients with bone disease, hypertension, and nephrolithiasis and age-matched showed a wide degree of overlap. However, when these PEA values were corrected for creatinine excretion, the group of patients with bone disease and with nephrolithiasis showed significantly greater amounts of PEA excreted by 80% and 44% respectively than did the control group. Serum calcium phosphorus values were different among these groups but serum alkaline phosphatase was significantly lower than control values in the patients with hypertension and nephrolithiasis.

PEA excretion in patients with endocrine dysfunction: Patients with certain endocrine disorders tended to excrete significantly more PEA than the age-matched control for the group. In acromegaly and adrenogenital syndrome, both the absolute amount of urinary PEA and the amount corrected for creatinine was significantly greater than their respective age-matched group.

Significance: These studies show that phosphoethanolamine excretion can no longer be considered pathognomonic for the disease hypophosphatasia. Variation in normal physiology and diet significantly alter daily excretion of the substance. In addition, the increased amounts of PEA excreted in various bone diseases, hypertension, and different endocrine disorders implies that a fundamental process common to many diseases results in the increased loss of PEA.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-01827-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Adverse effects of dietary protein on calcium balance in normal volunteers and patients with osteoporosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. A. Licata	Staff Fellow	HE NHLI
OTHER:	F. C. Bartter	Chief, Hyper-Endo. Branch	HE NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Since diets high in protein are often prescribed for patients with osteoporosis and can cause hypercalciuria in normal subjects, a study was undertaken to investigate the alteration in calcium balance induced by dietary protein in patients with osteoporosis and in normal volunteers. With the high protein diet, the mean urinary calcium of normals increased from 144 to 259 mg per day; that of the osteoporotics from 93 to 187 mg per day. Calcium absorption did not change with increased dietary protein in the normals; it rose by 12% with the high protein diet in the osteoporotics. Mean calcium balance was more negative with the high protein diet in both groups. It is concluded that excessive dietary protein has a marked effect on urinary calcium excretion and may be a factor in promoting negative calcium balance in osteoporosis.

PHS-6040
(12-75)

Project No.Z01-HL-01827-01 HE

Project Description:

Despite increasing evidence which has shown that added dietary protein increases urinary calcium excretion, high protein diets have been prescribed throughout the years to help combat this deficiency of bone matrix seen in osteoporosis. Because of these interrelated problems, we have investigated the effects of increased dietary protein on calcium balance in 5 normal volunteers and 3 patients with osteoporosis.

Methods Employed: Individuals were studied by metabolic balance and received a control diet of 0.5 gm protein/kg Bw with constant calcium, phosphorus, and sodium. This diet was supplemented with gluten, casein, gelatin and lactalbumin to provide a total protein intake of 2.0 gm/kg Bw.

Major Findings: Normals and patients with osteoporosis displayed an increase in urinary calcium loss when dietary protein was increased. This rise in calcium excretion was consistently found in everyone and occurred with 24-48 hours. The magnitude of the increase varied from person to person but averaged 77% in the normal group (900-1200 mg intake) and 108% in the patients with osteoporosis (800-900 mg intake).

In both groups, the high protein diet produced a significant increase in calcium and magnesium excretion without changes in sodium loss. In addition, potassium excretion decreased. Urine acidity and creatinine clearance increased but was significantly elevated only in the normal group. Associated with these changes, was an increase in the filtered and absorbed load of all these electrolytes mentioned. Calcium was the only electrolyte to show a decrease in the percent absorption. The other electrolytes displayed no change in percent absorption.

As regards serum chemical values, only blood urea nitrogen increased significantly in both groups. Other serum chemical values did not show consistent changes in both groups. The group of normals displayed significant increases in serum magnesium and serum albumin while the group of patients showed a decrease in serum potassium and carbon dioxide. All these changes mentioned however were within the normal limits of our laboratory values.

The effect of protein on calcium absorption was investigated by isotopic techniques in most of our subjects during each diet period. This value was calculated from the amount of radioactivity present in the forearm after successive doses of calcium -47 had been given intravenously and orally. Variable effects of dietary protein on calcium absorption were seen in the normal volunteers. Two showed increases in absorption, two showed decreases, and one showed no change. (The mean percent absorption during the low protein diet was 35% and during the high protein diet it was 39%). The two patients tested, however, showed striking increases in calcium absorption (which averaged 35% and 53% during the low and high protein diets, respectively).

The overall results on calcium balance were variable. Two volunteers showed a moderate increase in balance. The remainder however, showed decreases in calcium balance to less positive or negative values. The mean value of all balances was +106 mg/day (LP) and +38 mg/day (HP). The effect of protein on the calcium balance in our patients with osteoporosis are as follows: Each individual displayed a decrease in calcium balance when given the high protein diet. The mean balance was +60 mg/day during the low protein diet whereas during the high protein diet it was -40 mg/day. Different therapeutic manuevers were tried to correct the negative balance produced during the high protein diet. Hydrochlorothiazide medication was given to one male patient and partially corrected the negative balance induced by the added dietary protein. This change was probably attributable to the 90% decrease in urinary calcium excretion produced by the thiazide.

In a woman conjugated estrogens were given. This treatment only partially corrected the negative balance produced by the high protein diet. This change was done however without any appreciable change in urinary calcium excretion.

In another woman an additional 500 mg of elemental calcium was given daily during the high protein diet. This amount of calcium was 4-5X greater than the urinary calcium loss during the high protein diet. We noted that this additional calcium failed to change the negative balance induced by the high protein diet.

When estrogens was given in addition however, the balance became more positive but still did not completely reach the level found during the low protein diet.

Significance: We conclude that excess dietary protein can produce a negative calcium balance. Part of this negative effect may be attributable to increased urinary loss of calcium, secondary to altered renal function such as changes in filtered load of calcium, and the reabsorption of calcium from the urine.

However, part of this negative balance may result from an effect on the bone itself. Since added dietary calcium failed to correct the negative balance which resulted from the excessive urinary calcium excretion produced by the high protein diet, it would seem that bone itself might be playing a part in this overall process. How protein may induce loss of calcium from bone is not specifically known at present. It seems clear that excess dietary protein may be harmful to patients with osteoporosis rather than beneficial.

Publications:

1. Licata, A. A. and Bartter, F. C.: Adverse effects of dietary protein on calcium balance in patients with osteoporosis. Amer. College of Physicians, Philadelphia, PA, April, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01828-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pituitary-gonadal-adrenal interactions in patients with congenital adrenal hyperplasia and bilateral testicular tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Radfar	Staff Fellow	HE NHLI
OTHER:	R.J. Sherins	Senior Investigator	RR NICHHD
	F.C. Bartter	Chief, Hyper.-Endo. Br.	HE NHLI
	J. Kolins	Staff Fellow	LP NCI
	R. Easley	Staff Fellow	RR NICHHD
	N. Javapour	Senior Investigator	SURG NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

One patient with congenital adrenal hyperplasia and bilateral testicular tumors was studied. The light microscopy and electron microscopy of the tumor were consistent with "Leydig's cell tumor". During control period 24-hour urinary 17-KS, pregnanetriol, and plasma 17-OH-progesterone were elevated. The plasma LH was suppressed in the afternoon samples. The diurnal variation of plasma testosterone was absent. After 48 hours of dexamethasone administration, there was a resumption of periodic pulses of LH (every 90 minutes), and each peak was followed by a peak of plasma testosterone concentration 30 minutes later. These data suggest that adrenal androgens had resulted in suppression of LH during control period.

The spermatic vein plasma cortisol was three times peripheral vein level (14.3 mg/dl vs. 5.2) indicating that these testicular tumors secrete cortisol in spite of their morphologic resemblance of Leydig's cells.

Dexamethasone administration resulted in reduction in testicular size, while ACTH enlarged the testes, indicating that these tumors had ACTH dependent cells.

PHS-6040
(12-75)

Project No. Z01-HL-01828-01 HE

Project Description:

Objectives: The nature of bilateral testicular tumors associated with congenital adrenal hyperplasia has been a subject of controversy primarily because of great similarity between cytologic characteristics of what have been called 'adrenal rests and Leydig cells' and therefore the need for functional evaluation of such tumors is apparent. This fact led us to start studying such patients from a pathologic as well as functional point of view.

Methods: Those patients with congenital adrenal hyperplasia who are found to have testicular tumors will be admitted to the ward. They will have the routine evaluation; then an open testicular biopsy will be performed, followed by sequential blood sampling during a control period in which they will receive their usual dose of hydrocortisone and florinef. This period will be followed by sequential sampling after 48 hours of complete ACTH suppression (0.5 mg every 6 hours). The ability of their testicular tumors to secrete cortisol will be studied by blood sampling from the spermatic vein and a peripheral vein simultaneously during a period in which they are given ACTH 40 units every 12 hours I.M. in addition to their hydrocortisone and florinef.

Major Findings:

So far one patient has been studied. The light microscopy and electron microscopy of the tumor were consistent with "Leydig's cell tumor". During control period 24-hour urinary 17-KS, pregnanetriol, and plasma 17-OH-progesterone were elevated. The plasma LH was suppressed in the afternoon samples. The diurnal variation of plasma testosterone was absent. After 48 hours of dexamethasone administration, there was a resumption of periodic pulses of LH (every 90 minute), and each peak was followed by a peak of plasma testosterone concentration 30 minutes later. These data suggest that adrenal androgens had resulted in suppression of LH during control period.

The spermatic vein plasma cortisol was three times peripheral vein level (14.3 mg/dl vs. 5.2) indicating that these testicular tumors secrete cortisol in spite of their morphologic resemblance to Leydig's cells.

Dexamethasone administration resulted in reduction in testicular size, while ACTH enlarged the testes, indicating that these tumors had ACTH dependent cells.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-HL-01829-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Effect of nightly administered dexamethasone on HGH secretion in patients with CAH

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Radfar	Staff Fellow	HE NHLI
OTHER:	K. Trangle	Guest Worker	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.3	1.2

SUMMARY OF WORK (200 words or less - underline keywords)

In the therapy of congenital adrenal hyperplasia midnight administration of dexamethasone could have the advantage of lowering the risk of short stature (which occurs in many of such patients) by suppressing the production of adrenal androgens for longer periods. The question is whether such therapy may also result in inhibition of nocturnal release of growth hormone which is so important for growth. The results of our study should be able to answer this question. We have, so far, studied four pubertal children (2 boys and 2 girls)and two adult females. The results are pending.

PHS-6040
(12-75)

Project No.Z01-HL-01829-01 HE

Project Description:

Objective: In the therapy of congenital adrenal hyperplasia (CAH) the long acting cortisone analogues have the advantage of suppressing ACTH for a longer time, thereby lowering the risk of short stature which may result from the over-production of the androgens. The effect of dexamethasone will be increased if it is given at the time when ACTH is at its nadir i.e. at midnight. The nightly administration of dexamethasone in patients would be ideal only if it does not inhibit nocturnal secretion of growth hormone. This hypothesis led us to study the effect of a nightly administered, physiologic dose of dexamethasone on growth hormone secretion in patients with CAH.

Methods: Patients with salt-losing or non-salt-losing 21-hydroxylase deficiency CAH are admitted. During one period they receive their physiologic dose of dexamethasone at 7:00 a.m. They continue on this regimen for five days. On day five the patient is given an L-Dopa induced HGH stimulation test. On the night of day six, blood samples are drawn through an indwelling venous period, dexamethasone is administered at 11:00 p.m. and the studies performed during the first period are repeated.

Major Findings:

In the therapy of congenital adrenal hyperplasia midnight administration of dexamethasone could have the advantage of lowering the risk of short stature (which occurs in many of such patients) by suppressing the production of adrenal androgens for longer periods. The question is whether such therapy may also result in inhibition of nocturnal release of growth hormone which is so important for growth. The results of our study should be able to answer this question. We have, so far, studied four pubertal children (2 boys and 2 girls) and two adult females. The results are pending.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01830-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The effect of combined ACTH and HGH administration on aldosterone secretion.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. Radfar	Staff Fellow	HE NHLI
OTHER:	K. Trangle	Guest Worker	HE NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

If HGH does mediate ACTH effect on aldosterone production, supplementation of HGH should prevent the characteristic decline in aldosterone production seen after 2-3 days of ACTH administration alone. So far four males and females normal volunteers have finished the study. The data are being analyzed at this time.

PHS-6040
(12-75)

Project Description:

Objectives: Adrenocorticotropin (ACTH) stimulates the production of aldosterone, as shown by in vitro and in vivo studies. However, ACTH induces only a short term stimulation of aldosterone secretion, characterized by a return to control and below within 2-3 days in spite of continuous ACTH administration. This pattern of aldosterone secretion with ACTH stimulation is independent of sodium balance, potassium balance or renin status. The mechanism accounting for decline in aldosterone production after the second to third day, in spite of continued ACTH administration, is unknown. However, there is some evidence which suggests that HGH may be necessary for the stimulation of aldosterone secretion by ACTH: 1) In patients with panhypopituitarism administration of ACTH alone does not produce the initial significant increase in aldosterone production, 2) In hypophysectomized dogs, there is a decreased responsiveness of aldosterone production to ACTH. Normal responsiveness and continued aldosterone secretion with ACTH administration does occur in these dogs if growth hormone is given. This suggests a permissive role of HGH. These facts led us to investigate the role of HGH as a permissive factor in aldosterone secretion in man.

Project Protocol: The study was performed on normal volunteers; some will on 9 mEq Na diet, others on 249 mEq Na diet. Observation of blood electrolytes, supine and upright PRA, creatinine, DOC and aldosterone are made twice, during a 7 day control period, every other day during 6 days of administration of 40 units α1-24 corticotropin and 2 I.V. of HGH every 12 hours and every other day during 5 days following discontinuation of ACTH and HGH administration. Daily 24 hour urines will be collected for Na, K, creatinine, 17OHCS, 17KS, aldosterone and DOC.

Major Findings: If HGH does indeed mediate ACTH effect on aldosterone production, supplementation of HGH should prevent the characteristic decline in aldosterone production seen after 2-3 days of ACTH administration alone. So far four males and females normal volunteers have finished the study. The data are being analyzed at this time.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-01831-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less) "Studies in the SHR:"
Vascular protein synthesis of the spontaneously hypertensive rat.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Nakada	Visiting Scientist	HE NHLI
OTHER:	W. Lovenberg	Chief, Sec. of Biochem. Pharm.	HE NHLI
	F. C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

SUMMARY OF WORK (200 words or less - underline keywords)

Young spontaneously hypertensive rats (SHR) were either treated with hydralazine or hexamethonium or splanchnecotomized, so that development of hypertension was effectively arrested for two weeks. The rate of incorporation of ^{3}H-lysine into noncollagenous proteins in vivo of the heart, aorta and mesenteric arteries was determined in the treated SHR as well as in control SHR and in normal Wistar/Kyoto (WK) rats. The lysine incorporation into the noncollagenous protein of mesenteric arteries was increased in 8-week-old SHR as compared to that of WK rats. The elevated lysine incorporation in the SHR was abolished by treatment with hexamethonium or by splanchnecotomy, but was not affected by treatment with hydralazine. It is suggested that neutral innervation is important for the increase synthesis of vascular noncollagenous protein during the early hypertensive phase in the SHR.

PHS-6040
(12-75)

Project Description:

Of the great advantages of the spontaneously hypertensive (SHR) is that the entire life history of the disease's is compressed into a period of less than 2 years. The SHR resembles primary hypertension in man especially with respect to the increased peripheral vascular resistance. Evidence that high blood pressure causes an increased synthesis of collagen and elastin in the aorta of the hypertensive rat at the age of 15 months has accumulated from experimental studies. The blood vessels have been exposed to hypertension for prolonged time. Therefore, the elevated synthesis seen at this age could be explained only by the high blood pressure. Although no clear pathogenic factor has yet been identified with the development of hypertension, the peripheral vascular resistance in SHR appears to be increased at the initial stage of hypertension by a neurogenic mechanism that is subsequently replaced by a non-neurogenic mechanism. Neurogenic factors indicated by serum dopamine-β-hydroxylase activity and sympathetic discharge become similar to those of normotensive rats after hypertension is established.

In order to clarify the relationship between intensified neural activity and vascular protein metabolism, we studied the effect of pharmacological or surgical denervation of the mesenteric artery on ^{3}H-lysine incorporation into the collagen, noncollagenous protein and elastin of the heart and vasculatures in young SHR. The object of these experiments was to examine the potential interaction of the neurogenic and non-neurogenic mechanisms in the pathogenesis of hypertension.

Methods Employed: Male SHR and age-matched Wistar/Kyoto (WK/N) rats were used throughout the study. They were randomly divided into groups of about 10 animals at the age of six weeks and were given the following treatments: group I, nontreated SHR (10 rats), group II, hydralazine-treated (80 mg/L in drinking water) SHR (10), group III, hexamethonium-treated (50 mg/kg S.C. twice daily SHR (9), group IV, splanchnecotomized SHR (10) and group V, nontreated WK (8).

Animals received I.V. injection with 0.4 mCi/g of ^{3}H-lysine. They were sacrified by decapitation 2 hours after the injection. This time period appeared to be optimal as demonstrated in a previous study. The method for protein extripation is summarized in Chart I.

Major Findings: Final results of the antihypertensive treatments were shown in Table I. Lysine incorporation into the noncollagenous proteins of the heart and vasculature is shown in Table II. The only signficant difference between SHR and WK/N rats 8 weeks after birth was an increase in incorporation in the mesenteric arteries in SHR. Incorporation of ^{3}H-lysine into the noncollagenous protein of mesenteric arteries of the SHR was significantly lowered by hexamethonium treatment or splanchnecotomy. In these 2 groups the incorporation was similar to that in normotensive group. Conversely, hydralazine did not reduce the rate of incorporation. These differences were obvious both in the absolute values or when the values corrected for the lysine

specific activity.

While the incorporation of lysine into the collagenous protein of the aorta and mesenteric arteries was somewhat higher in the untreated SHR, the differences were of only marginal significance.

No major differences in incorporation into elastin are seen.

Publications:

1. Yamori, Y., Nakada, T. and Lovenberg, W.: Preliminary experiment of lysine work. Europ. J. Pharmacol, In press (1976).

Table I: Blood pressure and Organ Weights in Experimental Groups of Rats

Experimental Groups (No. of Rats)		Blood Pressure (mm Hg)	ORGAN WEIGHT		
			Heart (g)	Aorta (mg)	Nesenteric Arteries (mg)
I	Nontreated SHR (10)	168 ± 2	1.19 ± 0.05	126 ± 10	47 ± 2
II	Hydralazine-treated SHR (10)	136 ± 3**	1.30 ± 0.04	113 ± 6	48 ± 3
III	Hexamethonium-treated SHR (9)	137 ± 7**	0.80 ± 0.05**	108 ± 8	46 ± 3
IV	Splanchnicotomized SHR (10)	136 ± 3**	1.05 ± 0.05	106 ± 8	53 ± 2
V	Nontreated WK (8)	131 ±2.0**	0.91 ± 0.04**	98 ± 7	37 ± 1*

*P < 0.01

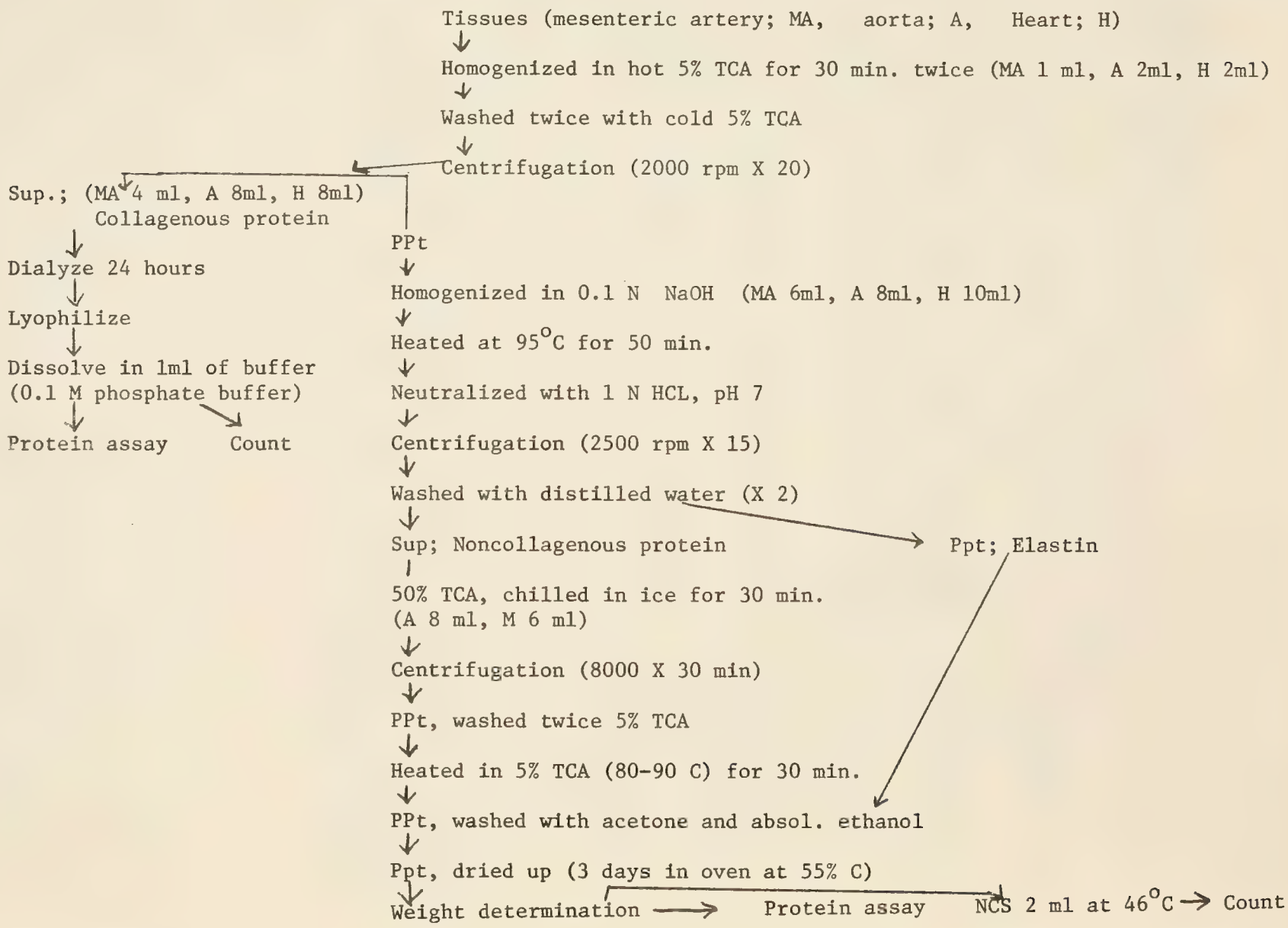
**P < 0.001

Table II: Incorporation of ^{3}H-lysine into noncollagenous protein in treated and nontreated SHR and WK

Experimental Groups (No. of Rats)		Heart	Aorta	Mesenteric Arteries
		dpm/mg Protein		dpm/100 mg Wet Weight
I	Nontreated (SHR (10)	394 ± 30	168 ± 45	1374 + 23
II	Hydralazine-treated SHR (10)	428 + 26	184 ± 56	1517 ± 152
III	Hexamethonium-treated SHR (9)	431 ± 13	245 ± 69	824 ± 95*
IV	Splanchnicotomized SHR (10)	395 ± 41	261 ± 53	790 ± 29*
V	Nontreated WK (8)	390 ± 5	201 ± 16	777 ± 48*

The tissues were removed 2 hours after injection of ^{3}H-lysine and the noncollagen protein extracted as described in the text. Values significantly different from the group I are indicated as follows: * $P < 0.001$. The free lysine specific radioactivity (dpm/mg) were as follows for each group: I 1066 ± 25, II 1067 ± 21, III 845 ± 57, IV 765 ± 72, and V 955 ± 100.

Chart I. Method For Protein Extripation Project No. Z01-HL-01831-01 HE



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01900-03 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Prostaglandins in Renal and Vascular Physiology.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert E. Bowden, M.D.	Staff Associate	HE-NHLI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE-NHLI
	David Horwitz, M.D.	Senior Investigator	HE-NHLI
	John J. Pisano, Ph.D.	Head, Sec. on Physiol. Chem.	CH-NHLI
	Lauren M. Cagen, Ph.D.	Staff Fellow	CH-NHLI
	John R. Gill, Jr., M.D.	Senior Investigator	HE-NHLI
	Frederic C. Bartter, M.D.	Branch Chief	HE-NHLI
	Addison A. Taylor, M.D.	Senior Investigator	HE-NHLI
	Nezam Radfar, M.D.	Pediatrician	HE-NHLI
	Robert P. Kimberly, M.D.	Clinical Associate	A&R-A
	Paul H. Plotz, M.D.	Senior Investigator	A&R-A
	Teruhiro Nakada, M.D.	Guest Worker	HE-NHLI

COOPERATING UNITS (if any)
Laboratory of Chemistry, NHLI
Arthritis and Rheumatism Branch, NIAMDD

LAB/BRANCH
Hypertension-Endocrine

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	3.0	2.5

SUMMARY OF WORK (200 words or less - underline keywords)

The role of prostaglandins in renal physiology remains unclear. In the last year we have shown that there is a circadian rhythm for urinary prostaglandin E excretion (iPGE) in normal women and that this is an essential consideration in the design of any acute experiments. Alterations of posture at the same time of day reveal a significant fall in urinary iPGE excretion when the subject stands. Alterations of dietary sodium and potassium intake had no effect on urinary iPGE excretion. These procedures have been shown to have a pronounced effect on the renin-angiotensin-aldosterone system. Therefore, iPGE excretion may be independent of the renin-angiotensin-aldosterone system in normals. Aspirin administration to patients with lupus nephritis caused a fall in glomerular filtration rate which parallels the fall in urinary prostaglandins. This suggests that prostaglandins may have an important role in regulation of glomerular filtration rate in patients with lupus nephritis. Prostaglandins are important in the pathophysiology of Bartter's syndrome. Urinary iPGE excretion is elevated in patients with this syndrome and treatment with prostaglandin synthetase inhibitors reversed the abnormalities of the syndrome including hyperreninemia and hypokalemia. The exact mechanism of action of prostaglandins in this disorder is as yet unexplained.

737

Project Description:

Objectives: 1) Further development of methodology for measuring prostaglandins (PG) in subnanogram range in biologic fluids, and 2) application of these methods to the study of the role of prostaglandins in renal physiology and in the pathology of hypertension.

Methods: Radioimmunoassays have been developed, as reported previously by this laboratory, which allow reliable measurements of PGA and PGE with a sensitivity of 150 to 200 pg.

Major Findings: A. Humans

1. iPGE excretion in normal subjects. The range of urinary excretion of immunoreactive prostaglandin E (iPGE) is 50 $\pm$ 20 ng/hr (mean $\pm$ SD) for normal women eating a 109 mEq sodium and 100 mEq potassium diet.

a) Our study in 6 normal women indicates that there is a circadian rhythm for urinary prostaglandin excretion with a nadir at 3 am to 7 am and an apex at 3 pm to 11 pm with an amplitude of +30% ($p < .001$). The rhythm is not affected when the subject changes from ad lib activity to 24 hours of bed rest. The circadian rhythm of iPGE excretion parallels the rhythms of urinary potassium, sodium, volume, and creatinine.

b) Urinary iPGE excretion decreases from 57 $\pm$ 21 to 39 $\pm$ 16 ng/hr when the subject changes from supine to erect posture ($p < .01$) if the time of day is controlled; i.e., the subjects are either supine or upright during the same time interval on successive days. Urinary potassium, volume, sodium, and creatinine also fall significantly.

c) When sodium intake was changed from 9 mEq to 259 mEq in three subjects there was no change in iPGE excretion in either the first three days or after one week of high salt intake. Similarly when the dietary intake of potassium was changed from 185 mEq to 25 mEq daily in 8 subjects there was no change either acutely or chronically (up to 9 days) in urinary iPGE excretion.

d) Reduction of dietary fluid intake in one subject from 2500 cc to 1000 cc caused a 40% decrease of urinary iPGE excretion and an equivalent decrease in urine volume that returned to control as dietary fluid was increased back to control.

2. iPGE excretion in hypertensive patients. Nine hypertensive women have been studied on a controlled 109 mEq sodium and 100 mEq potassium diet. Each subject's excretion of iPGE was within two standard deviations of normal. There was no significant difference between the normals and the hypertensive subjects.

a) In order to further investigate the mechanism controlling PGE excretion, one patient with a pheochromocytoma was studied under control conditions, with phenoxybenzamine treatment, and with alpha-methyl-paratyrosine treatment.

There was no change from control in urinary iPGE excretion with either of these treatments. A second patient with primary aldosteronism was studied while normokalemic on a 9 mEq sodium diet, while hypokalemic on a 109 mEq sodium diet, and while normokalemic with spironolactone. There was a 20% decrease in urinary iPGE excretion when the subject changed from the low salt to the normal salt diet but no change noted with spironolactone.

b) Three hypertensive patients were treated with indomethacin for one week. Indomethacin had no effect on their blood pressure. The angiotensin II receptor blocker (P-113), which has mild agonist activity, was given to one patient both before and during indomethacin treatment. The infusion of P-113 did not lower the blood pressure, instead it increased the blood pressure. The increase in blood pressure to P-113 was increased markedly by indomethacin administration.

3. iPGE excretion in patients with lupus nephritis. Three patients have been studied before, during, and after receiving therapeutic doses of aspirin for control of systemic lupus erythematosis (SLE). Aspirin therapy produced a marked increase in serum creatinine from 0.9 $\pm$ .2 to 1.3 $\pm$ .2 mg/dl and a corresponding decrease in inulin clearance. These returned to control levels with cessation of aspirin. One of these three patients had an elevated urinary iPGE level of 135 ng/hr which was suppressed 45% by aspirin therapy. The other two patients had values of iPGE excretion greater than 1 standard deviation above normal and these suppressed 45 and 60%, respectively, with aspirin.

4. Study of the effects of PG synthetase inhibition in patients with Bartter's syndrome. Nine patients with Bartter's syndrome have been studied. The mean excretion of iPGE was 145 $\pm$ 30 ng/hr with seven of the nine patients having supranormal values. Treatment with indomethacin produced a 60 $\pm$ 10% decrease in urinary iPGE excretion along with a marked decrease in urinary sodium and potassium excretion, and in plasma renin activity. Serum potassium rose rapidly. There were highly significant correlations in each individual between the excretion of iPGE and renin, urinary potassium and serum potassium. Two individuals had an initial rise followed by a decrease in serum potassium during therapy. These two individuals also had an increase in urinary iPGE excretion during therapy which corresponded to the fall in serum potassium. A second inhibitor, either Ibuprofen or aspirin, was used in three patients. There was less suppression of iPGE excretion with these two agents when compared to indomethacin; i.e., 40 $\pm$ 5% suppression, and also a marked attenuation of the other changes noted with indomethacin treatment.

5. Occurrence of PGA and PGA metabolites in urine. Urine samples have been extracted and chromatographed for PGA and then radioimmunoassayed. Human urine contains 100 to 1000 pg/ml of prostaglandin A-like material. This iPGA is not formed by the extraction procedure. If free prostaglandins are extracted from urine and discarded and the urine is then treated with methanolic KOH, a substance remains which co-chromatographs with PGB and reacts with antibody to PGB. This substance is decreased by treatment with indomethacin.

B. Dogs

1. Effects of indomethacin in conscious dogs with stenosis of the left renal artery. This is a continuation of studies reported previously. Female dogs were prepared with bilateral ureterostomies and 75% occlusion of the left renal artery by means of a surgical ligature. At least two weeks later, when the animals had recovered and hypertension had developed, renal clearance studies were performed while the animals were awake. Urine volume, clearances of PAH, inulin and free water, and excretions of iPGE and iPGF were all significantly lower on the stenotic side as compared to the normal side. Administration of indomethacin intravenously (doses of 2 to 10 mg/kg) caused an 80% decrease in prostaglandin excretion and a significant increase in urine volume, sodium excretion, and free water clearance for both sides. The study has been extended this year to include a series of control infusions with the vehicle (0.1M Na_3PO_4 buffer, pH 7.5, in 10% ethanol). These infusions of vehicle can account for only about 50% of the changes produced by the infusions of indomethacin plus vehicle.

2. Infusion of PGE into the renal artery of anesthetized dogs. PGE_2 was infused at rates of 10 ng to 100 ng/min into the left renal artery of anesthetized dogs. Urine was collected during 40 minute control, infusion and post-infusion periods and analyzed for iPGE, sodium and potassium. There was a progressive fall in iPGE excretion throughout the experiment. If longer control periods (120 min) were used then there was a stabilization of iPGE excretion and no change when PGE_2 was infused. Indomethacin decreased iPGE output by 80%, and again PGE_2 infusion caused no change in urinary iPGE as measured by radioimmunoassay. Urinary sodium increased 35% in most animals during the infusion and returned to control levels when the infusion was discontinued. Samples of urine to which some of the infusion mixture was added revealed a $\pm$ 15% deviation from the predicted value.

3. Injection of ^{3}H-PGA into the left renal artery of dogs. Two µCi of ^{3}H-PGA_1 was injected into the left renal artery of anesthetized dogs and samples of renal venous blood and urine were obtained. Three polar metabolites have been found in the urine which may represent analogs of the material found in human urine, i.e. metabolites of prostaglandin A.

C. Rodents

1. Effects of indomethacin and PGA infusions on prekallikrein in spontaneously hypertensive rats (SHR) and Wistar/Kyoto (W/Ky) control rats. Indomethacin (4 mg/kg) administered by gastric lavage for three days significantly reduced serum prekallikrein levels in both SHR and W/Ky rats ($p < .05$). Prekallikrein was also significantly reduced 30 minutes after intravenous injection of indomethacin at 2 mg/kg ($p < .05$) in both groups. PGA_1 injected I.V. at 12 µg/100 gm body weight raised prekallikrein significantly in the SHR ($p < .05$) but not in the W/Ky rats.

Significance to Biomedical Research and Institute Program: The control of prostaglandin excretion is still poorly understood. The data presented here

show that there is a circadian rhythm for the excretion of urinary prostaglandins and that the circadian rhythm is not affected by a change in posture, which is known to affect the circadian rhythms for renin and angiotensin. This implies that prostaglandins may be independent of the renin-angiotensin system. Urinary prostaglandin E is not affected by alteration of sodium or potassium intake, however, some preliminary results indicate that there may be a direct correlation between iPGE excretion and urinary volume. This suggests an interaction with vasopressin. Prostaglandin levels fall when a subject stands and again this is opposite of the effects of the same maneuver on renin and angiotensin. The occurrence of the circadian rhythm is important especially in the design of any acute experiment in which prostaglandins will be measured.

The elevation of urinary prostaglandins in patients with Bartter's syndrome may explain the pathophysiology of this syndrome. The reversal of all the abnormalities of the syndrome by indomethacin strongly supports this interpretation. When other less potent inhibitors of PG synthetase are used, there is a smaller reduction in urinary iPGE and lesser changes in the other components of the syndrome. It is not certain whether prostaglandins are the primary abnormality causing a response in the renin-angiotensin system or a secondary abnormality responding to an abnormality in the renin-angiotensin system. However, the lack of marked changes in the aldosterone excretion in these patients when treated with indomethacin implies that aldosterone may not account for the tremendous potassium excretion and hypokalemia in these patients.

The finding of supranormal levels of urinary prostaglandin in patients with lupus nephritis is very preliminary. It may reflect the degree of disease activity or it may indicate that prostaglandins are involved in the regulation of renal blood flow. The suppression of urinary iPGE by aspirin correlates well with the fall in GFR.

In summary, prostaglandins have been shown to be important in renal physiology. They may regulate renal blood flow in certain disease states as well as interact with the renin-angiotensin system in other disease states. However, the exact control mechanisms for their excretion remain to be elucidated.

Proposed Course of Project: 1. Study of PG excretion in patients with various types of hypertension before and during treatment. 2. Studies of PGE excretion in patients and normals in whom the renin-angiotensin system is either stimulated or inhibited by either physiologic or pharmacologic means. 3. Study the effects of indomethacin and other PG synthetase inhibitors in patients with Bartter's syndrome. 4. Study further the interrelationships between prostaglandins, the kallikrein-kinin system, and the renin-angiotensin system. 5. Study the effects *in vivo* of prostaglandins and antidiuretic hormone.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01902-07 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Investigations of the Sympathetic Nervous System in Human Hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Horwitz, M.D.	Senior Investigator	HE NHLI
OTHER:	Walter Lovenberg, Ph.D.	Head, Section on Biochemical Pharmacology	HE NHLI
	Michael F. Roizen, M.D.		LCS-NIMH
	Horst Grobecker, M.D.		LCS-NIMH

COOPERATING UNITS (if any)

Laboratory of Clinical Science, National Institute of Mental Health

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Section on Experimental Therapeutics

INSTITUTE AND LOCATION

National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	.55	.55

SUMMARY OF WORK (200 words or less - underline keywords)

Norepinephrine and dopamine-β-hydroxylase (DβH), which are released when sympathetic nerves are stimulated, were studied as indicators of sympathetic nerve activity. Mean plasma norepinephrine levels were higher in 28 sustained or intermittent hypertensives than in matched normotensives (hypertensives 0.25, normotensives 0.18 ng/ml, $p < 0.02$); mean plasma levels of DβH activity were 42 and 35 units respectively ($p > .27$) in the same subjects. Plasma DβH activity showed a wide scatter in both groups and correlated poorly with either plasma norepinephrine levels or diastolic blood pressure. DβH activity in plasma of seven patients with secondary hypertension was indistinguishable from levels in the other groups. Plasma levels of norepinephrine doubled after the subjects had been erect for ten minutes whereas plasma DβH activity, corrected for small changes in the concentration of plasma proteins, remained unchanged. It is concluded that plasma DβH activity is an insensitive index of sympathetic nerve function; the findings suggest increased sympathetic nerve activity in the subjects with essential hypertension.

PHS-6040
(12-75)

Project Description: Project No.: Z01 HL 01902-07 HE

Studies of Plasma Dopamine-β-hydroxylase (DβH) Activity and Norepinephrine Levels in Hypertensive and Normotensive Subjects.

Objectives: Dopamine-β-hydroxylase (DβH) catalyzes the final step in the synthesis of norepinephrine, the sympathetic neuromediator. DβH is released from sympathetic nerves, can be measured in plasma and has been proposed as an index of sympathetic nerve activity in man. It has been reported that plasma DβH levels are higher in patients with essential hypertension than in subjects with primary etiologies for their hypertension and that such differences are of diagnostic value. Our past observations suggested that plasma levels of DβH were an insensitive indicator of sympathetic nerve activity and that the wide range of values impaired their usefulness for judging sympathetic activity in individual subjects. Our recent studies were intended to determine whether sympathetic nerve activity differs in hypertensives in comparison with subjects with normal blood pressure or secondary hypertension. The usefulness of measurements of DβH activity was evaluated by comparison with an established and sensitive indicator of sympathetic nerve activity, i.e., norepinephrine levels.

Methods: Twenty-eight patients with sustained (20) or intermittent (8) essential hypertension were matched for age, sex, and race with normotensive subjects. Blood pressure was determined in supine and erect postures on eight separate visits to establish mean values and variability for each subject. After these familiarization visits subjects underwent blood sampling via an indwelling needle in an arm vein; samples were drawn after 30 and 40 minutes of rest and two and ten minutes of standing. Plasma DβH activity was determined by the Nagatsu method utilizing the enzymatic conversion of tyramine to octopamine and photometric assay after oxidation of octopamine to p-hydroxybenzaldehyde. Plasma norepinephrine was measured by the method of Roizen et. al.; the norepinephrine was enzymatically N-methylated and tritium-labeled through use of [3H-methyl]-S-adenosylmethionine and phenylethanolamine-N-methyltransferase; the radioactive product was extracted and assayed by liquid scintillation spectrometry. Mean values were compared using the Wilcoxon matched-pairs signed ranks test; computation of correlations utilized the Spearman rank correlation coefficient.

Major Findings: Mean levels of plasma norepinephrine while subjects were resting were significantly higher among hypertensive than normotensive subjects (0.25 versus 0.18 ng/ml, respectively, $p < 0.02$); corresponding values for DβH activity were 42 and 35 units but the wide scatter precluded statistical significance ($p > .27$). Levels of norepinephrine were limited to a 3- to 5-fold range among different subjects whereas the range for DβH extended from 0 to 128 units per ml. The correlation of DβH activity with either norepinephrine levels or diastolic blood pressure was weak (Spearman rank correlation coefficients of 0.12 and 0.10, respectively). Mean plasma levels of norepinephrine increased by 97% among hypertensive and 98% among normotensive subjects after 10 minutes of quiet standing; corresponding increases for DβH activity were 8 and 7% but these increases disappeared after correction for increases in the concentrations of plasma proteins during standing (Δ + 8%). Seven patients with secondary hypertension (hyperaldosteronism 3, renovascular 3, polycystic

kidneys and systemic lupus erythematosis 1) showed mean levels (32 units) and a range (3-72) of DβH activity which were indistinguishable from those of the other groups.

Significance to Biomedical Research and the Program of the Institute: The findings are compatible with an increased level of activity of the sympathetic nervous system in patients with essential hypertension. Plasma DβH activity is shown to be an insensitive index of sympathetic nervous system function in man.

Proposed Course: The half-life of plasma dopamine-β-hydroxylase will be studied in subjects with high and low levels of dopamine-β-hydroxylase.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01903-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Plasma Prekallikrein and Kininogen in Hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Izzo, M.D.	Staff Associate	HE NHLI
OTHER:	J.M. Vinci, M.D.	Staff Associate	HE NHLI
	D. Horwitz, M.D.	Senior Investigator	HE NHLI
	H.R. Keiser, M.D.	Deputy Chief	HE NHLI
	J.J. Pisano, Ph.D.	Head, Section on Physiological Chemistry	CH NHLI

COOPERATING UNITS (if any)
Laboratory of Chemistry, National Heart and Lung Institute

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.25	.25

SUMMARY OF WORK (200 words or less - underline keywords)

Prekallikrein (PPK) and total kininogen (KGN) were determined in human plasma. PPK was determined by radiochemical esterolytic assay, KGN by radioimmunoassay of bradykinin after trypsin digestion of plasma. In normal volunteers, no changes in PPK or KGN were effected by changes of posture, by infusions of vasoactive amines, or by variation of dietary sodium. Patients with essential hypertension and with Bartter's Syndrome have normal values of PPK, but may have supranormal values of KGN. In a patient with renal artery stenosis, KGN values from the affected side were lower than values from the normal kidney, with PPK values equal.

PHS-6040
(12-75)

Project Description:

Objectives: Despite our demonstration of subnormal values of urinary kallikrein in patients with essential hypertension and supranormal values in patients with primary aldosteronism, the role of the plasma kallikrein-kinin system in health and disease remains to be elucidated. The purpose of this project is to further expand knowledge of plasma prekallikrein (PPK) and total plasma kininogen (KGN) as they relate to physiologic manipulations and pathological conditions.

Methods: PPK was determined by the radiochemical esterolytic assay of Beaven and Pisano. Values are expressed in TAME (p-tosyl-L-arginine methyl ester) units per ml plasma. (One TAME unit (TU) is the amount of enzyme required to hydrolyze 1 μmole TAME per minute at pH 8 and 30°). Previous data from this laboratory employed partially purified plasma prekallikrein as standard. Present data refers to a human urinary kallikrein (HUK) standard, and is felt to allow easier standardization and comparison of data. HUK was kindly supplied by Dr. J. Pierce, NHLI.

Total plasma kininogen (KGN) was determined by radioimmunoassay of bradykinin generated by trypsin digestion of whole plasma. It is expressed in μg/ml plasma and μg/g protein (the latter referring to Lowry analysis of total plasma protein).

Ten normal volunteers were studied during variations of daily Na+ intake, postural changes, and with intravenous infusion of vasoactive amines. Steady-state infusion of amines for (2) 1-hour periods followed a 5% dextrose control period. Bloods were collected each period. Each amine was infused in sufficient dosage to achieve a measurable cardiovascular effect: norepinephrine and phenylephrine, >20 mm increase in diastolic BP; isoproterenol, >30 beat/min increase in heart rate; dopamine, >20 mm Hg increase in pulse pressure. 3-Hour infusions of 5% dextrose and 0.9% NaCl were further controls.

Hypertensives (n=17) on ad lib diets, were also studied, as were patients with Bartter's Syndrome (n=5), and renal artery stenosis (n=1).

Major Findings: A. Plasma prekallikrein (PPK) (values expressed as mean ± S.E.).

1) There were no differences in PPK in patients on 109 meq Na+ intake (1.3 ± 0.1 TU/ml, n=3) who were then changed to 259 meq Na+ intake (1.2 ± 0.1 TU/ml, n=3). No difference in PPK was observed in patients changed from 109 meq Na+ intake (1.47 ± 0.16 TU/ml, n=4) to 9 meq Na+ intake (1.47 ± 0.15 TU/ml, n=4). These findings do not support previous data showing increased PPK on low Na+ diets.

2) No significant effect of posture on PPK was observed: supine, 1.22 ± 0.10 TU/ml, n=12; standing, 1.29 ± 0.10 TU/ml, n=12.

3) Vasoactive amines (norepinephrine, phenylephrine, isoproterenol and

dopamine) in moderately active doses had no effect on PPK values in normal volunteers (n=3).

4) Hypertensive subjects, some on therapy, had PPK values (1.50 ± 0.10 TU/ml, n=17) different from normal controls (1.15 ± 0.10 TU/ml, n=7). However, the age differences do not permit fair comparison of these groups. Also, these results do not support previous data of lower PPK values in hypertensives compared to normals.

5) Patients with Bartter's Syndrome had PPK values (1.40 ± 0.10 TU/ml, n=5) no different from hypertensives. No effect on PPK was observed during therapy with indomethacin or ibuprofen.

6) One patient with renal artery stenosis was studied with blood samples from selective renal venous catheterization. No differences were observed between the normal and affected side.

B. Kininogen (values expressed as mean ± S.E.).

1) Normals on 109 meq Na+ intake had total kininogen values of 2.90 ± 0.16 µg/ml plasma or 40 ± 6 µg/g protein. The same subjects on 259 meq Na+ had 2.58 ± 0.20 µg/ml or 35 ± 4 µg/g protein, n=3. On 9 meq Na+ intake, 4 normal volunteers had total kininogens of 3.06 ± 0.16 µg/ml or 43 ± 4 µg/g protein. The difference between the values in µg/g for 9 and 259 meq Na+ diets were not statistically significant, but the number of subjects studied was small.

2) No significant effect of posture on total kininogen was observed: supine, 3.78 ± 0.20 µg/ml or 53 ± 6 µg/g protein; standing 3 hours, 3.87 ± 0.22 µg/ml or 47 ± 5 µg/g protein, but the difference was not statistically significant.

3) Vasoactive amine infusions as previously described had no measurable effect on total kininogen.

4) Hypertensive subjects were studied, some on therapy, with males 3.56 ± 0.22 µg/ml or 56 ± 2 µg/g protein (n=9) and females 4.54 ± 0.25 µg/ml or 65 ± 3 µg/g protein (n=4). Although the difference between males and females in KGN expressed in µg/ml is significant ($p < .01$), the values in µg/g protein are not statistically significant. Also the number of subjects is small. (Normals have 3.78 ± 0.22 µg/ml or 53 ± 6 µg/g protein total KGN).

5) Females with Bartter's Syndrome had KGN 4.60 ± 0.32 µg/ml or 69 ± 4 µg/g protein. These values were not significantly different from the group of female hypertensives studied, but were significantly higher ($p < .01$) than the normals.

6) In a patient with renal artery stenosis, the affected side had lower (3.68 µg/ml) KGN than the IVC (5.10 µg/ml) or the contralateral normal kidney (4.78 µg/ml). However, the same figures corrected for total plasma protein gave 73, 69 and 76 µg/g protein, respectively.

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Significance to Biomedical Research and the Program of the Institute: The kallikrein-kinin system appears to have two separate branches: plasma and glandular. The relationship between the two branches and the physiologic significance of each remains unclear.

Within the plasma system, PPK is probably present in huge excess over active plasma kallikrein. Similarly, total circulatory kininogen represents at least 1000 times the amount of circulating bradykinin. Although PPK and KGN measurements may poorly reflect the overall activity of the kallikrein kinin system, they may be useful benchmarks for further studies. Certain conditions, such as Bartter's Syndrome and immune-complex inflammatory disorders may be associated with abnormal levels of kininogen.

Proposed Course: 1) Establish a race, sex and age-adjusted range of normal values for PPK and KGN.

2) Obtain more data on the effects of variations of dietary Na+ have on KGN, in hypertensive subjects (especially those with renal artery stenosis) and patients with Bartter's Syndrome.

3) Study plasma KGN in patients with a systemic inflammatory disease, e.g., systemic lupus erythematosis.

4) Develop a specific assay for high molecular weight kininogen (the preferred substrate for PPK) or the fragments of kininogen degradation. This would allow better assessment of the overall activity of the plasma kallikrein-kinin system.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01905-06 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Urinary and Plasma Kallikrein and Kinin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.M. Vinci, M.D.	Staff Associate	HE NHLI
OTHER:	D. Horwitz, M.D.	Senior Investigator	HE NHLI
	H.R. Keiser, M.D.	Deputy Chief	HE NHLI
	J.J. Pisano, Ph.D.	Head, Section on Physiological Chemistry	CH NHLI
	P.J. Cannon, M.D.		

COOPERATING UNITS (if any)
Laboratory of Chemistry, National Heart and Lung Institute
Department of Medicine, Columbia University

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	3

SUMMARY OF WORK (200 words or less - underline keywords)

Urinary kallikrein and kinin and plasma bradykinin were measured in normal subjects and in patients with hypertension, Bartter's syndrome, and scleroderma on controlled intakes of sodium and potassium. Plasma bradykinin in normal subjects is responsive to changes in posture and sodium intake and parallels changes in the renin-angiotensin-aldosterone system. Urinary kallikrein increases with sodium restriction but urinary kinin excretion is unchanged. Urinary kinin excretion is significantly correlated with kallikrein excretion on 109 meq sodium intake but not on 9 meq sodium intake. This suggests that urinary kallikrein is not the only determinant of urinary kinin excretion. Patients with Bartter's syndrome have significantly elevated basal urinary kallikrein and plasma bradykinin but subnormal urinary kinin excretion. During prostaglandin synthetase inhibition urinary kallikrein and plasma bradykinin decreased while urinary kinin excretion increased toward normal. These data indicate an involvement of the kallikrein-kinin system in this syndrome and its relationship to other vasoactive systems. Patients with scleroderma but without hypertension or apparent renal disease have subnormal urinary kallikrein excretion which may be related to reduced renal blood flow in these patients.

PHS-6040
(12-75)

Project Description:

Objectives: To study the role of the plasma and urinary kallikrein-kinin system in normal subjects, and in patients with hypertension with and without renal disease.

Methods: Urinary kallikrein excretion is measured with a previously described radiochemical assay [NHLI-79(c)].

Urinary kinin is measured by a radioimmunoassay developed in this lab with an antibody which binds bradykinin, kallidin and methionyl-lysyl-bradykinin, and thus gives a measure of total immunoreactive kinins.

Plasma bradykinin is processed for radioimmunoassay by a modified method of Talamo and Spragg.

Urinary kallikrein and kinin and plasma bradykinin were measured in normal subjects and in patients with hypertension, Bartter's syndrome or scleroderma. Most studies were performed with the subject on a controlled intake of sodium and potassium. This was necessary because we have demonstrated previously that the excretion of kallikrein in the urine is directly responsive to the level of circulating sodium-retaining steroid.

Major Findings:

1. Relationships to sodium intake:

Normal man: Eight normal subjects on an intake of 109 sodium and 100 meq K daily showed a significant increase in plasma bradykinin after three hours of upright position (2.80 ng/ml $\pm$ 0.91 (mean $\pm$ S.D.) to 4.18 $\pm$ 1.11, $p < 0.02$). When sodium intake was reduced to 9 meq/day there were significant elevations of: a) urinary kallikrein (7.0 tu/d $\pm$ 3.5 to 18.5 $\pm$ 7.3, $p < 0.005$), b) supine plasma bradykinin (2.80 ng/ml $\pm$ 0.91 to 6.02 $\pm$ 2.90, $p < 0.01$) and c) upright plasma bradykinin (4.18 ng/ml $\pm$ 1.11 to 8.47 $\pm$ 4.28, $p < 0.02$). Urinary kinin excretion was not significantly altered by the change in sodium intake (11.11 µg/day $\pm$ 5.26 to 12.08 $\pm$ 6.95). Maximum stimulation of plasma bradykinin occurs with sodium depletion and upright posture and is highly significantly different from values during 109 meq Na intake and supine posture (2.80 ng/ml $\pm$ 0.91 to 8.47 $\pm$ 4.28, $p < 0.005$). While urinary kinin excretion is significantly correlated with urinary kallikrein excretion in subjects on a Na intake of 109 meq, there is no significant correlation between urinary kallikrein and urinary kinin excretion on a 9 meq sodium intake. This suggests that urinary kallikrein is not the only determinant of urinary kinin excretion. These findings support possible relationships between the kallikrein-kinin system and the renin-angiotensin aldosterone system.

2. Bartter's Syndrome:

Patients with Bartter's syndrome on 109 meq Na and 270 meq K intake have significantly elevated basal urinary kallikrein excretion (mean $\pm$ SEM) (23.4 tu/d $\pm$ 2.7) compared to normal subjects (7.3 $\pm$ 1.0, $p < 0.001$), elevated su-

pine plasma bradykinin (9.98 ng/ml ± 1.85) compared to normal subjects (2.94 ± 0.26, $p < 0.005$) but subnormal urinary kinin excretion (5.80 µg/d ± 1.02) compared to normal subjects (12.77 ± 1.31, $p < 0.005$). During prostaglandin synthetase inhibition with either indomethacin or ibuprofen urinary kallikrein decreased to 12.97 tu/d ± 4.90, $p < 0.001$, plasma bradykinin decreased to 3.65 ng/ml ± 1.44, $p < 0.005$, and urinary kinin excretion increased to 12.87 µg/d ± 1.95, $p < 0.001$. With prostaglandin inhibition, there was Na and K retention, correction of hypokalemia and suppression of renin and aldosterone. These data indicate an involvement of the kallikrein-kinin system in Bartter's syndrome, and several strong interrelationships between the kallikrein-kinin system, the renin-angiotensin system and prostaglandins. In particular they indicate a possible negative feedback of PGE excretion on kinin excretion.

3. Scleroderma:

Seven patients with scleroderma but with neither hypertension or apparent renal disease on ad lib sodium intakes had significantly lower kallikrein excretion 3.2 ± 1.7 (mean ± S.D.) than normal subjects on 109 meq Na intake (7.3 ± 3.7) ($p < 0.02$). This may relate to our previous finding that urinary kallikrein excretion is correlated with renal blood flow. Standing plasma bradykinin was significantly elevated (9.39 ng/ml ± 5.09) compared to that of control subjects (4.30 ng/µl ± 2.09, $p < 0.001$). Urinary kinin excretion was not significantly different from that of control subjects.

Significance to Biomedical Research and the Program of the Institute: These data continue to support our hypothesis that the kallikrein-kinin system is involved in salt and water homeostasis and in the hormonal response to changes in posture. Furthermore, the data show a strong relationship between the kallikrein-kinin system, renin-angiotensin aldosterone system, and prostaglandins.

Proposed Course: 1) Further study of the interrelationships of the kallikrein-kinin system, the renin-angiotensin system and prostaglandins. This will involve measurement of components of both the urinary and plasma kallikrein system in a) patients with primary aldosteronism, b) normal and hypertensive subjects on variable sodium and potassium intakes and with fludrocortisone, c) hypertensive subjects on low and normal salt intakes before and during prostaglandin synthetase inhibition, d) hypertensive subjects on low and normal salt intakes given the angiotensin converting enzyme inhibitor SQ 20881, e) normal subjects given infusions of angiotensin I, angiotensin II and prostaglandins, and f) normal subjects on 9 meq Na intake given a 48-hour ACTH infusion.

2) Further characterize the kallikrein-kinin system in hypertensives under normal and stimulatory conditions.

3) Define the relative role of the kallikrein-kinin system in antagonizing ADH.

4) Relate the reduction in renal blood flow (with Xenon flow studies) and the degree of renal involvement in scleroderma to the kallikrein-kinin system.

Publications:

Keiser, H.R., Andrews, M.J., Guyton, R.A., Margolius, H.S. and Pisano, J.J.: Urinary kallikrein in dogs with constriction of one renal artery. Proc. Soc. Exp. Biol. & Med. 151: 53-56, 1976.

Keiser, H.R., Geller, R.G., Margolius, H.S. and Pisano, J.J.: Urinary kallikrein in hypertensive animal models. Fed. Proc. 35: 199-202, 1976.

Margolius, H.S., Horwitz, D., Pisano, J.J. and Keiser, H.R.: Relationships among urinary kallikrein, mineralocorticoids and human hypertensive disease. Fed. Proc. 35: 203-206, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01906-03 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Urokinase Excretion and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Izzo, M.D.	Staff Associate	HE NHLI
OTHER:	V. Hial, M.D., Ph.D.	Visiting Fellow	HE NHLI
	J.J. Pisano, Ph.D.	Head, Section on Physiological Chemistry	CH NHLI
	D. Horwitz, M.D.	Senior Investigator	HE NHLI
	H.R. Keiser, M.D.	Deputy Chief	HE NHLI

COOPERATING UNITS (if any)

Laboratory of Chemistry, National Heart and Lung Institute

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Section on Experimental Therapeutics

INSTITUTE AND LOCATION

National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.50	

SUMMARY OF WORK (200 words or less - underline keywords)

Urokinase (UK, urinary plasminogen activator) excretion (UKE) was assayed by radiochemical esterolytic assay in human urine. In normal volunteers, upright posture increased UKE and decreased urine kallikrein excretion and urine flow, when compared to supine posture. This suggested a possible relationship of UKE with sympathetic nervous system activity. No effect on UKE was observed during dietary variation of sodium and potassium, or during infusion studies with vasoactive amines. No diurnal variation of UKE was observed. The presence of mucoprotein and the formation of cold precipitates which change UK in many urines necessitates assay of fresh samples. The origin of urinary kinins was investigated using chromatography and bioassay. Human urinary kallikrein generated lys-bradykinin and bradykinin. Met-lys-bradykinin (MLBK) is largely artifactual, resulting from the action of uropepsin on urokininogen in acidified urine. MLBK is not found in urines to which the uropepsin inhibitor pepstatin has been added.

PHS-6040
(12-75)

Project Description:

Objectives: Urokinase (UK, urinary plasminogen activator, E.C. 3.4.99.26), a limited protease which cleaves plasminogen to form plasmin, has been demonstrated in human urine by fibrinolytic (fibrin plate) and esterolytic assays. It is thought to function in the maintenance of tubular potency by lysis of fibrin; no other function has been demonstrated.

The relationship of exercise and posture to increased plasma fibrinolysis and preliminary data suggesting an association of upright posture with increased UK excretion has led to speculation that UK excretion may reflect sympathetic nervous system activity. It is also known that injection of vasoactive amines will increase plasma fibrinolysis; to these ends, vasoactive amines were infused into normal volunteers to determine their effects on UK excretion. The possible role of UK in the generation of urinary met-lys-bradykinin was also investigated.

Methods: UK was assayed by the radiochemical esterolytic assay developed by Pisano, et al., with results expressed in CTA units (Committee on Thrombolytic Agents, NIH) per unit time.

Three normal volunteers received infusions of vasoactive amines. Each amine was infused in sufficient dosage to achieve a measurable cardiovascular effect: norepinephrine and phenylephrine, >20 mm increase in diastolic BP; isoproterenol, >30 beat/min increase in heart rate; dopamine, >20 mm increase in pulse pressure. Each amine was infused on a separate day with a control infusion of D5W for the first hour followed by the amine infusion in D5W for 2 hours. These results were compared with 3 hour control infusions of either D5W or normal saline. Inulin and PAH were added to all infusions for determination of RBF and GFR. Urokinase excretion was determined in urine samples collected during each hour of the infusions.

The effect of posture on UK excretion was studied in 12 normal volunteers (6 men and 6 women). UK excretion, urine kallikrein excretion, and urine volumes were determined for the 4-hour interval from 5 to 9 am. Subjects were either supine or ambulatory on alternate days of the 4-day study. Diurnal variation was also assessed in supine patients' urines collected in 6 consecutive 4-hour periods. Urine kallikrein was determined by bioassay. Results were expressed in kinin units (KU) standardized against known amounts of lys-bradykinin.

The origin and content of met-lys-bradykinin (MLBK), lys-bradykinin (LBK) and bradykinin (BK) were studied. Identification of MLBK was based on: (1) chromatographic separation using IRC-50, SP-Sephadex C-25, and bioassay using isolated guinea pig ileum or rat uterus; (2) conversion by dipeptidyl aminopeptidase I (DAP-I) to BK (also established by chromatographic and bioassay procedures); and (3) amino acid composition. Pepstatin, a potent pepsin inhibitor, was also employed. 24-Hour urine specimens were obtained from 17 normal volunteers, age 18 to 48.

Major Findings: (1) Previous findings about the stabilization of UK samples by NaCl and protein were reconfirmed. However, it was noted that the formation of cold-induced urine precipitates (attributed to the presence of urinary mucoprotein) markedly impaired the accuracy of the UK assay. The resulting inhomogenous mixture caused 100% variation in repeated assays of some samples; in others, especially those of low activity, all esterase activity could be found in association with the precipitate. It is therefore recommended that all assays be performed on fresh urine. Also because of the low activity of some samples, larger aliquots should be assayed to achieve appropriate sensitivity.

(2) Recent data on 24-hour UK excretion (7900 ± 3900 CTAU/day, mean ± S.D., n=5) reconfirms previous data of Wilcox. It is noted that while these are relatively consistent values for each individual, the huge variation between individuals (range 2100 to 16,500 CTAU/24 hrs) makes interpretation difficult.

(3) Dietary variation of Na^+ (9 to 259 meq/24 hrs) and K^+ (25 to 185 meq/24 hrs) had no effect on UK excretion.

(4) Amine infusions (norepinephrine, phenylephrine, isoproterenol, and dopamine) in moderate pressor doses had no effect on resting UK excretion (6.1 ± 3.1 CTAU/min, mean ± S.D., n=3).

(5) UK excretion was found to increase significantly ($p < .005$) when supine values (737 ± 177 CTAU/4 hrs, mean ± S.D.) were compared to upright values (1245 ± 195 CTAU/4 hrs). During the same period, urine volume fell significantly ($p < .001$) when supine values (377 ± 56 ml/4 hrs) were compared to upright values (156 ± 26 ml/4 hrs). A 24% fall in 4-hour urine kallikrein excretion ($p < .05$) was observed in upright compared to supine position.

(6) No diurnal variation in UK excretion was observed.

(7) Three kinins were demonstrated in human urine: BK, LBK, and MLBK. Mean values were (in µg/day) (mean ± S.D.):

	BK	LBK	MLBK
Men	3.4 ± 0.6	7.6 ± 1.1	9.5 ± 1.6
Women	2.5 ± 0.7	5.1 ± 1.0	1.2 ± 0.4

LBK is considered the principal kinin generated from low molecular weight kininogens by urinary kallikrein, while BK is the result of the action of plasma kallikrein on high molecular weight kininogen. The nature of the MLBK fraction was elucidated from chromatographic and bioassay procedures; it was found only in urine collected without pepstatin. One subject demonstrated 90% reduction in 24 hr MLBK when pepstatin was added to the collection vial. Pepstatin did not alter the excretion of BK or LBK. Addition of purified kininogen to acidified urine from a subject with congenital absence of kininogen led to the formation of MLBK but no BK or LBK.

The conclusion reached is that in urine acidified after excretion, MLBK is formed by the action of uropepsin. These results, combined with the finding that human plasmin generates only BK from human kininogen suggest that UK has no apparent role in the generation of urinary MLBK.

Significance to Biomedical Research and the Program of the Institute: The function of UK remains to be elucidated. The possible relationship of UK to sympathetic nervous activity is obscured by the large variation from individual to individual and by the failure of moderate doses of vasoactive amines to affect UK excretion. However, it is possible that only near-maximal sympathetic nerve stimulation will result in consistently increased UK excretion (as has been shown by Rosing, et al., for plasma fibrinolysis).

Demonstration of the formation of MLBK by uropepsin clarifies in part the role of urinary kallikrein in generation of LBK (and BK). Presence of MLBK appears to be largely artifactual.

Proposed Course: Studies will be performed to establish the relationship between plasma and urinary plasminogen activators. These will include studies of patients being treated with UK for thrombotic disorders, and physiologic studies correlating plasma fibrinolysis and UK excretion in normal volunteers.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01907-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Catecholamine Metabolites in Normal and Hypertensive Subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.L. Izzo, M.D.	Staff Associate	HE NHLI
OTHER:	C.R. Zimmerman, M.S.	Research Assistant	CH NHLI
	H.R. Keiser, M.D.	Deputy Chief	HE NHLI
	J.J. Pisano, Ph.D.	Head, Section on Physiological Chemistry	CH NHLI

COOPERATING UNITS (if any)

Laboratory of Chemistry, National Heart and Lung Institute

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Section on Experimental Therapeutics

INSTITUTE AND LOCATION

National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.75	

SUMMARY OF WORK (200 words or less - underline keywords)

Gas chromatography (GC) with flame-ionization detection has been used to measure the catecholamine metabolites homovanillic acid (HVA), vanillylmandelic acid (VMA), and methoxyhydroxyphenyl glycol (MHPG) in human urine. Gas chromatography-mass spectrophotometry has been used to verify the purity of GC peaks obtained. Present methods are simpler than existing methods which employ electron-capture detection or mass fragmentography.

Project Description:

Objectives: It has long been reasoned that the nervous system plays a major role in the development of essential hypertension. The purpose of this study is to assess the importance of catecholamine metabolites (especially homovanillic acid and methoxyhydroxyphenyl glycol) in physiologic changes in normals and in pathologic conditions, including hypertension. To this end, refinement of existing gas chromatographic methods has been pursued to give an easy and reproducible assay system.

Methods: 1) Dopamine (DA) and homovanillic acid (HVA) can both be measured by gas chromatographic (GC) methods. We have refined previously published methods for HVA using acid-extracted human urine, trimethyl silyl (TMS) derivatization, and flame-ionization (FI) detection. Methods for internal standardization are currently being perfected. Vanillylmandelic acid (VMA) can be quantitated simultaneously. DA technology remains to be developed.

2) Methoxyhydroxyphenyl glycol (MHPG) can also be quantitated by GC. Our method is a modification of existing methods requiring hydrolysis of urinary MHPG sulfate, neutral extraction, TMS derivatization, and FI detection. An internal standard will be added to each sample.

Major Findings: In several samples, HVA, VMA, and MHPG have been identified as separate GC peaks. Two samples have been confirmed by GC-mass spectroscopy to give pure peaks using our present methodology.

Significance to Biomedical Research and the Program of the Institute: Current evidence from several lines links CNS and sympathetic nervous activity to hypertension. Vascular resistance, cardiac output, and the renin-angiotensin system are influenced by sympathetic activity. Plasma catecholamines are modestly elevated (50%) in essential hypertension, but the range of values is large.

Many technical problems have retarded research into the complex role of the CNS in essential hypertension. 1) Sympathetic dysfunction has not been proven to be a cause of essential hypertension. 2) No overall measure of sympathetic nervous activity exists. 3) Changes in the metabolism of a single CNS region may be overshadowed by the CNS metabolism as a whole, especially when CSF, urine or plasma values are used to approximate CNS metabolism. 4) Methods for segregating central from peripheral nervous effects are not perfected. 5) A measurable physiologic response is generally mediated through changes at several levels within the nervous system. 6) It is often difficult to determine whether CNS changes are primary or secondary in relation to an observed event. 7) Human tissue (CNS) is generally unavailable for study; and 8) animal models are of limited value in human essential hypertension.

Human urinary MHPG has been shown by Goodwin et al., to result largely from CNS norepinephrine metabolism. MHPG excretion correlates well with levels of both systolic and diastolic pressure in normals. It may therefore be a useful measure of sympathetic nervous activity.

HVA is the major metabolite of DA. DA is not only important in the CNS, it is probably responsible (at least in part) for ganglionic control of sympathetic nervous activity, and it is also found in the kidney. Urinary DA has been found to correlate positively with natriuresis. DA agonists have also been implicated in hormone release phenomena, particularly prolactin and aldosterone. Therefore, measurement of the excretion of MHPG and HVA may provide valuable insights about CNS involvement in essential hypertension.

Proposed Course: Urinary homovanillic acid (HVA), vanillylmandelic acid (VMA) and methoxyhydroxyphenyl glycol (MHPG) will be assayed in normotensive and hypertensive subjects. Variation of dietary sodium intake, postural changes, and exercise may be used to elucidate differences between normals and hypertensives. The effects of various catecholamine blocking agents, agonists, and antihypertensive drugs on catecholamine metabolites will be studied.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01908-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Prostaglandin Biosynthesis and the Role of Prostaglandins in Cardiovascular Physiology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.M. Zusman, M.D.	Clinical Associate	HE NHLI
OTHER:	H.R. Keiser, M.D.	Deputy Chief	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

SUMMARY OF WORK (200 words or less - underline keywords)

Renal medullary interstitial cells have been isolated from rabbits and grown in tissue culture. These cells exhibit characteristic lipid containing vacuoles on light and electron microscopic examination. These cells in tissue culture produce large quantities of prostaglandins E and F, as measured by radioimmunoassay. Prostaglandin E production is stimulated by arachidonic acid, bradykinin, angiotensin II, anti-diuretic hormone, and hyperosmolarity. Prostaglandin production is decreased by potassium, acetyl salycylic acid, indomethacin, ibuprofen and meclofenamic acid.

760

PHS-6040
(12-75)

Project Description:

Objectives: To study the biosynthesis of prostaglandins by renal medullary interstitial cells grown in tissue culture.

Methods: 1) PG Biochemistry. Standard techniques of column and thin-layer chromatography have been employed to isolate arachidonic acid, and prostaglandins A, E and F for further quantitative analyses.

2) Radioimmunoassay. Prostaglandins in plasma, as well as buffer are measured by RIA using modifications of the techniques previously developed by the principal investigator and in this laboratory.

Major Findings: Renomedullary interstitial cells have been isolated from rabbit renal medulla and successfully grown in tissue culture. These cells can be trypsinized and serially passed in culture. Evaluation of the cells by light microscopy reveals a fibroblastoid morphology with characteristic lipid containing vacuoles. Electron microscopic examination confirms the presence of lipid containing granules in the cytoplasm.

The cells produce large quantities of prostaglandins of the E, and F series. The production of PGE is greatest during the log phase of cell growth and decreases as the cells reach confluence. In short term incubation studies in buffer systems it has been found that PGE production increases in response to bradykinin, arginine vasopressin, and angiotensin II; the addition of arachidonic acid markedly stimulates PGE and PGF production. Inhibition of PGE production has been observed after the addition of acetylsalicylic acid, indomethacin, meclofenamate, and ibuprofen. Hyperosmolarity of the cell buffer is also a potent stimulant of PG production.

Significance to Biomedical Research and the Program of the Institute: Although prostaglandins are produced by a large number of cell systems, little information about the regulation of PG biosynthesis is available in homogenous cell populations. The role of prostaglandins in renal physiology is particularly difficult to ascertain because of hemodynamic effects in the kidney which cannot be eliminated in the *in vivo* system. The tissue culture system we have developed will allow for the study of pharmacologic stimuli to prostaglandin biosynthesis free of hemodynamic factors. Furthermore, the mechanism of cell stimulation will be capable of study in such a system.

Proposed Course: 1) The mechanism of stimulation of prostaglandin biosynthesis in renal interstitial cells grown in tissue culture.

Having demonstrated that renal medullary interstitial cells grown in tissue culture produce prostaglandins, studies will be done to investigate the mechanism of synthesis stimulation.

2) The role of prostaglandins in ACTH stimulated adrenal function.

Although a good deal of evidence from *in vitro* studies exists for the role of prostaglandins in ACTH stimulated adrenal steroidogenesis, little is known about the *in vivo* role of the prostaglandins in the adrenal. The performance of ACTH infusion tests after prostaglandin synthesis inhibition will help to elucidate the physiologic role of prostaglandins in the adrenal.

3) The effect of a prostaglandin E_2 infusion on the renin-angiotensin-aldosterone, and kallikrein-kinin systems in normal and hypertensive subjects.

PGE_2 infusion studies will evaluate the metabolism of PGE_2 in man. In addition we will attempt to confirm a recent report that PGE_2 administration results in *de novo* prostaglandin A_2 synthesis. Potential alterations in the renin-angiotensin-aldosterone, and kallikrein-kinin systems during the infusion will help to elicidate the relationship of these two systems.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01909-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Information Retrieval in Pharmacology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elise A. Brown, Ph.D.	Research Pharmacologist	HE-NHLI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE-NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

SUMMARY OF WORK (200 words or less - underline keywords)

Information retrieval and appraisal activities were established for the areas of biochemical pharmacology and toxicology. The project aids new research by determining the extent to which the subject has already been explored and by examining the available procedures for answering a question. The computer files of the National Library of Medicine, as well as the personal files and informed opinions of various investigators have been used.

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PHS-6040
(12-75)

Project Description:

Objectives: This project report was transferred from the Pulmonary Branch, NHLI, and was previously reported under project number Z01 HL 02516-01 PB. The purpose of this project is to facilitate the initiation of laboratory research projects by determining the present knowledge on a topic and by finding the methods necessary to solve the problem. Patient care physicians need knowledge of drug interactions and other toxicological data for their experimental projects.

Methods: The complex nature of modern medical research requires that both physical-chemical information and clinical information are readily available. Many of the procedures used in research are not standardized but are changed to suit the nature of the problem. Biomedical information is contained in the Medline, Toxline and other files of the National Library of Medicine. There has been considerable dissatisfaction with the limited coverage of the physical-chemical literature in the NLM data bases. I, therefore, initiated a contract with the Lockheed Corporation which has on-line 21 different files including:

1. Chemical Abstracts Condensates (Chemcon)
2. Scisearch - the Current Contents file
3. NAL/CAIN - complete bibliography of the National Agricultural Library
4. Biosis Previews - Biological Abstracts and BioResearch Index.

These files were not available previously at the NIH or NLM. All these files plus the personal files and expertise of the investigator in the area of biochemical pharmacology were used to aid investigators in their search for information.

Appended is a list of topics as requested by more than 31 investigators that have been explored.

1. Repeated requests for information on the following drugs: Indomethacin, Ibuprofen, Meclofenamic acid, Minoxidil, and Hydralazine.
2. Studies of prostaglandins and Ibuprofen or Indomethacin
3. Studies with hypertensive rats
4. Occurrence of cerebral hemorrhage with hypertension
5. Treatment of pheochromocytoma and carcinoid tumors
6. Systolic time intervals in congestive heart failure
7. The hypotensive effects of bradykinin and kallikrein on the kidney and the mediation of the effects by prostaglandins
8. Kininase II, complete bibliography
9. Angiotensin receptors

10. A complete bibliography on the familial and genetic aspects of coronary artery disease, especially angina pectoris. The investigator came to me when the library facilities were found to be unsatisfactory.

11. Interactions of acridines with RNA and RNA polymerase

12. Occurrence of manganese activated ATPase in mammalian systems

13. ATP, adenosine, and ATPase content of brain in convulsive disorders

14. Reviews of propranolol

15. Lung biochemistry

    Guanyl cyclase and adenyl cyclase content; cGMP and cAMP content
    ATP and ATPase levels
    Acetyltransferase content

16. Kidney metabolism

    Effect of isoproterenol and dopamine on kidney function tests, renal veins and blood flow in the kidney
    Adrenergic receptors in the kidney
    Transport systems in chicken kidney
    Toxicity of penicillin
    Guanyl cyclase and cGMP content
    Effects of paraquat
    Effects of cephalosporins and penicillins on kidney

17. Lipids

    Proteolipid structure
    Ion exchange chromatography of LDL lipoproteins
    Effect of neomycin on lipid metabolism
    Lipoprotein synthesis in aorta
    Effect of isoproterenol on pineal body lipid metabolism

18. Collagen biochemistry

    Biosynthesis in the lung and in the eye
    Effects of azothioprine on lung metabolism and collagen formation
    Effects of penicillin on liver collagen
    Interaction of colchicine with collagen synthesis and liver cirrhosis

19. The interrelationship of $[Ca^{++}]$ and the secretory rate in pancreas and parotid gland

20. The interrelationships between $[Ca^{++}]$, cyclic AMP, cyclic GMP and/or the two cyclase enzymes

21. Chemical methodology and characterization

The use of Raman spectroscopy in biology
Metabolism of lignocaine, lidocaine, and xylocaine
Determination of dopamine, methoxyhydroxyphenyl glycol
Aspirin and congeners
High speed liquid chromatography
Diglycerides; determination and characterization

22. Other searches dealt with topics such as:

Parenteral infusion of prostaglandin E in humans
Bartter's disease - etiology and treatment
Characteristics of aging in cells, i.e., enzyme variations and chemical composition
Biochemical pharmacology of fluoroamphenicol

Significance to Biomedical Research and Institute Program: The increased emphasis on medical research since World War II has generated a large mass of biomedical information. More efficient methods are needed for determining the current status of a problem. This project speeds up access to the medical literature. It saves the time of expensive personnel by more rapidly ascertaining whether a project has been done and by examining methods to obtain the most efficient way of answering a question.

Proposed Course of Project: It is proposed to increase the speed of access to computer files and to increase the number of files available to include more physical-chemical information. The demand for services has more than doubled this year over the last year. I suggest that a cathode ray tube terminal and high speed printer be obtained which operates at 120 cps to replace the current typewriter terminal which operates at 15 cps. This equipment would increase the efficiency of searches and, also, would reduce the noise level in our crowded space.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01910-03 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Biochemical Effects of Paraquat on the Lung

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Elise A. Brown — Research Pharmacologist — HE-NHLI

OTHER: Harriet M. Maling — Head, Section on Physiology — CP-NHLI

COOPERATING UNITS (if any)

Laboratory of Chemical Pharmacology, NHLI

LAB/BRANCH
Hypertension-Endocrine

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart and Lung Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The herbicide, paraquat (Pq), has been reported to decrease the amount of pulmonary surfactant (dipalmitoyl lecithin) in rat lung. We have found (Maling et al., Fed. Proc. 34:226, 1975) that a beta adrenergic blocking agent, propranolol (Pr), reduces about 50% the 48-hr mortality of rats from Pq (25 mg/kg, i.p.). Since Pr modifies phospholipid metabolism (Hauser and Eichberg, J.B.C., 250:105, 1975), we have therefore studied in vitro the effects of Pq, Pr, and Pq + Pr on the incorporation of ^{32}P-PO_4 by rat lung slices. Pq increased by 40% the amount of $^{32}PO_4$ incorporated in 30 min into phosphatidyl inositol (PI) and decreased the amount in phosphatidyl glycerol (PG) by 20%. Pr decreased the amount of ^{32}P in phosphatidyl choline (PC) by 70% and increased the amount in PG by 130%, in phosphatidic acid (PA) by 390%, in lyso-PA by 400%, in lyso-PC by 390%, and in cytidine diphosphate (CDP)-diglyceride by 1000%. The combination of Pq + Pr reduced ^{32}P in PG by 10%, in phosphatidyl ethanolamine by 50%, and in PC by 90%. Pq + Pr increased the amount of ^{32}P in PA by 500%, in PI by 160%, in lyso-PA by 460%, and in CDP-diglyceride by 780%. The amount of ^{32}P incorporated into dipalmitoyl lecithin was reduced 57% by Pq, 70% by Pr, and 94% by Pq + Pr. There were no significant differences in the total ATP content of the groups at the end of the incubation period. The in vitro changes do not explain the protective effects of propranolol in vivo.

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PHS-6040
(12-75)

Project Description:

This project report was transferred from the Pulmonary Branch, NHLI, and was previously reported under project number Z01 HL 02515-02 PB.

Objectives: Since pulmonary damage may be associated with changes in lung surfactant (dipalmitoyl lecithin, DPL), measurements were made of the effects of paraquat (Pq) (5×10^{-4}M), propranolol (Pr) (5×10^{-4}M) and Pq + Pr on the incorporation of ^{32}P-phosphate into the phospholipids (PL) of rat lung slices. Pr has been reported to alter the pattern of ^{32}P-phosphate incorporation into the PL of the pineal gland. Similarly, Pq has been reported to decrease the amount of DPL in rat lungs after 3 days (Fisher, Clements, & Wright, J. Appl. Physiol. 35:268, 1973). Lung slices were preincubated with Pq, Pr or Pq + Pr at 37°C for 30 minutes before the addition of ^{32}P-phosphate and the incubation was continued for another 30 minutes. Table 1 shows that there was a significant redirection in the flow of ^{32}P-phosphate through the PL classes in 30 minutes.

Table 1
Uptake of ^{32}P into Phospholipid Classes

Phospholipid Class	Control	Pq	Pr	Pr + Pq
	Ratio = Experimental/Control			
Phosphatidyl-glycerol	1.0	.8**	1.3**	0.9*
Phosphatidyl-ethanolamine	1.0	.8	0.6	0.5**
Phosphatidyl-choline	1.0	.8	0.3**	0.1**
Phosphatidic Acid	1.0	1.5	3.9**	5.0**
Phosphatidyl-inositol	1.0	1.4*	1.1	1.6*
Cytidine-diphospho-diglyceride	1.0	1.6	10.2*	7.8**
Lyso-phosphatidic acid	1.0	1.6	4.1**	4.6**
Lyso-phosphatidyl choline	1.0	1.0	3.9*	0.7

* = $p < .005$; ** = $p < 0.001$

The incorporation of ^{32}P was increased markedly in PL classes which are minor components of control slices. For example, phosphatidic acid, the lyso-compounds and CDP-diglyceride are almost undetectable except after drug treatment. Incorporation into phosphatidyl choline and phosphatidyl ethanolamine,

the major components of PL in control slices, was markedly reduced by Pq, Pr and Pq + Pr.

Table 2 shows that Pq, Pr, and Pq + Pr markedly decrease the incorporation of ^{32}P-phosphate into the anenoic lecithins (predominantly DPL). The decrease in DPL synthesis with Pq treatment is not surprising in view of the finding by some, but not all, investigators that paraquat *in vivo* decreases the amount of DPL in rat lungs. We were surprised that treatment with Pr also reduced DPL synthesis *in vitro*.

Table 2

Incorporation of $^{32}PO_4$ into Anenoic Lecithins

Parameter	Control	Pq	Pr	Pq + Pr
cpm/mg Lung slice	677	289	200	40
% Control	100	43	30	6

Levels of ATP were examined after *in vitro* incubation under the same conditions. Table 3 shows that each of the treatments decreased ATP content at 30 minutes, but the differences from the control slices were not significant at 60 minutes. These data cannot explain the protective effects of Pr on Pq mortality or the DPL data (Table 2) since these drugs appear to have similar effects on ATP content and DPL formation from $^{32}PO_4$. They are synergistic in some of these effects.

Table 3

ATP Content of Rat Lung Slices

Minutes at 37°C	Control	Pq	Pr	Pq + Pr
	Percent of Initial Content			
30	55	37	39*	30**
60	41	29	30	20

* = $p < 0.05$

** = $p < 0.001$ as compared with the appropriate control value

Significance to Biomedical Research and Institute Program: The pulmonary toxicity of paraquat in rats has been used as a model for pulmonary fibrosis

and for the sudden distress syndrome of the newborn. Pulmonary surfactant changes have been observed with both of these disorders. Elucidation of the mechanisms underlying these conditions may lead to treatments for these serious pulmonary diseases.

Proposed Course of Project: Isoproterenol (ISO) increases the toxicity of Pq for rats and this increase can be counteracted by Pr (Maling & Brown, Fed. Proc. 34:226, 1975). The effects of combinations of Pq, Pr, Pq + Pr, Pq + ISO, Pq + Pr + ISO on PL synthesis from ^{32}P-phosphate will be examined in rat lung slices. Similar uptake studies will be made using C^{14}-palmitic acid so that the acylation and deacylation reactions of PL may be assessed as well as the ^{32}P-phosphate-ATP-CTP reactions. ISO induces DNA synthesis and cell division in the salivary gland of the rat (Furuno & Matsudaira, Exptl. Cell Res. 94:431, 1975). It is proposed to examine the effects of Pq, Pr and Pq + Pr on the DNA synthesis of rat lungs.

Publications: None

ANNUAL REPORT

SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART AND LUNG INSTITUTE

The research of this section is largely devoted to the molecular mechanisms regulating neurotransmitter synthesis and to the molecular events that occur as transmitters impinge upon their target cells and organs. It is anticipated that this fundamental approach to neuronal regulation will help resolve the putative role for neuronal systems in blood pressure regulation and the pathogenesis of essential hypertension. With this objective in mind we have also established a research program utilizing the genetic rat models for hypertension and stroke and have developed collaborative clinical studies to explore neuronal factors in various hypertensive diseases. While many of our projects are integrated and overlapping with each other, progress of the past year will be presented under several headings proceeding from the most basic chemical studies to the physiological and clinical interpretations.

I Molecular Properties of Monoamine Biosynthetic Enzymes

Tyrosine-, dopamine-β- and tryptophan hydroxylase are three mixed function oxygenases that catalyze important steps in the synthesis of dopamine, norepinephrine and serotonin. These transmitters occur in specific neurons which have important regulatory functions.

Tyrosine hydroxylase. This enzyme is rate limiting in the synthesis of catecholamines and is the key regulatory point in their synthesis. Tyrosine hydroxylase has never been isolated in its native form, although other laboratories have isolated an active fragment after tryptic digestion. We have attempted to isolate the native enzyme from brain. By modification of standard protein fractionation techniques (ammonium sulfate and Sepharose 4B and hydroxyapatite chromatography) the enzyme has been substantially purified. The specific activity of these enzyme preparations are an order of magnitude greater than those reported in the literature, although analytical disc gels reveal several major protein bands in addition to tyrosine hydroxylase. While not totally successful in providing a pure brain tyrosine hydroxylase, this project has provided a very useful preparation for the regulatory studies.

Dopamine-β-hydroxylase (DBH). The final step in norepinephrine synthesis is catalyzed by this enzyme. In contrast to tyrosine hydroxylase, DBH can be isolated as a pure protein. In previous years we have described techniques for isolating pure and highly active DBH and have shown that this enzyme is a tetrameric glycoprotein. During the past year we have obtained further convincing evidence by finger print analysis that the subunits of this protein are identical and that an active dimeric form of the enzyme (MW 150,000) exists.

DBH contains at least one copper per monomer and it is thought that at least two copper atoms are required to form each active site. Using EPR to monitor the oxidation state of the copper it has been shown with redox couples that the tetrameric enzyme in the oxidized state can accept 4 electrons and that the redox potential of the active site is + 307 ± 12 mv. It has also been shown that the enzyme as isolated exists in a partially reduced state that can be fully oxidized upon the addition of the substrate, tyramine. Because of

this and the observed hyperfine splitting of the copper signal upon addition of substrate, it has been concluded that the substrate interacts directly with the copper active site. We have also found that ferrocyanide can replace ascorbate as the electron donor in the assay system thus eliminating the need for catalase. This system will greatly simplify the mechanism studies that are planned.

As a result of these studies we have formed a tentative conception of the DBH molecule as consisting of four glycoprotein subunits each containing a copper atom. Two subunits appear to cooperate to form an active site and the native enzyme would aappear to consist of two catalytically active dimers.

Tryptophan hydroxylase. This enzyme is the initial and rate limiting enzyme serotonin synthesis. Like tyrosine hydroxylase, tryptophan hydroxylase has yet to be isolated as a homogeneous active protein. Highly active preparations have been prepared by polyethylene glycol extraction and Sepharose chromatography. These preparations are not pure and because of the apparent extreme lability of the partially purified enzyme such a goal appears to be impossible at this time.

II Regulation of Transmitter Synthesis

The biosynthesis of the monoamine neurotransmitters discussed above is regulated by the first enzyme (tyrosine and tryptophan hydroxylase) in their biosynthetic pathways. These two enzymes appear to have a common reaction mechanism, each utilizing tetrahydrobiopterin as its cofactor.

In our previous report we pointed out that tyrosine hydroxylase can be activated by a cAMP dependent protein phosphorylation. This reaction causes a decrease in the K_m of the enzyme for its cofactor. Since the cofactor is present in limiting amount in neuronal tissue, this provides a rapid mechanism for adjusting the rate of transmitter synthesis. During the past year, the mechansim of this protein kinase activation of tyrosine hydroxylase has been further explored. No incorporation of ^{32}P from labeled ATP into the enzyme can be demonstrated by either immunochemical or sucrose gradient techniques. We have obtained evidence that the activation of the enzyme in vivo by treatment of animals with dopamine receptor blockers is similar to the activation by protein kinase. Mixing experiments with extracts of drug treated and control animals suggest that an activator molecule may exist. Such an activator is likely to be a phosphoprotein, since the activation by protein kinase is retained after passage of the enzyme mixture through a Sephadex column. The in vivo activation by dopamine receptor blockers is also retained following chromatography on Sephadex G-25. The existence of such a putative phosphoprotein would be consistent with the observation that similar activation of tyrosine hydroxylase occurs when other negatively charged polymers such as heparin and micells of phospholipids are added to the reaction mixture. A similar activation of the enzyme from the corpus striatum by EGTA and deactivation by calcium was also reported. While such a role for calcium would have important implications, extensive and carefully controlled studies have failed to establish any function of calcium in the activation-deactivation process. We have also found that little activation of highly purified enzyme occurs with protein phosphorylation, suggesting that an activator molecule may have been separated during the purification. The complete mechanism by which a cAMP dependent protein phosphorylation regulates

the synthesis of catecholamine is unknown, but rapid progress is being made toward this goal.

Less is known about the regulation of tryptophan hydroxylase activity. The major in vivo regulatory system appears to be substrate availability. While no direct end-product inhibition can be demonstrated, conditions which cause the accumulation of serotonin in tissue are known to reduce the synthesis rate of the amine. In a series of experiments we administered the monoamine oxidase inhibitor, phenelzine, to rats for up to 21 days and regional brain content of serotonin and tryptophan hydroxylase were measured. The serotonin content of brain regions rose rapidly within the first few hours of drug treatment but returned toward normal levels during the second and third weeks. In contrast to this apparent decrease in the in vivo activity, in vitro activity was significantly increased (20%) in the mesencephalic tegmentum. These experiments clearly demonstrate that factors other than substrate availability and end-product levels control the activity of tryptophan hydroxylase in the brain. These factors are under further investigation. During the past year we have also completed a study of the effects of p-chloroamphetamine and related compounds on the central serotonergic systems. A single dose (10 mg/kg) of the above drug causes up to a 50% loss of tryptophan hydroxylase for 3 months or longer. This dramatic and specific action of p-chloroamphetamine appears to be due to specific uptake and concentration within serotonergic nerve endings. Chlorphenteramine, a currently marketed appetite supressant and close chemical analogue of p-chloroamphetamine, was found to have no toxic effect on the serotonergic system.

Another means by which activity across a synapse can be regulated is by the re-uptake mechanisms. Detailed studies on the neuronal release and re-uptake of norepinephrine in heart slices and brain synaptosomes have indicated that an active transport system exists for the translocation of norepinephrine in both directions across the neuronal membrane. The data obtained in these studies is consistent with, but does not prove a role for ion gradient active transport in neuronal transmission.

III Neuronal Regulation of Protein Synthesis

In addition to the immediate response to nerve impulses, target cells in some cases exhibit a long term adaptive response. This long term response usually involves the selective increase in the rate of synthesis of one or more proteins. This section is currently studying three different systems in which this type of regulation appears to occur. These systems are: the transsynaptic induction of tyrosine hydroxylase in sympathetic ganglia and carotid body, the beta receptor dependent induction of serotonin-N-acetyltransferase in pineal, and the sympathetic dependent synthesis of non-collagen protein in the mesenteric arteries.

Transsynaptic induction of tyrosine hydroxylase. In our previous reports we described several of the factors that control transsynaptic induction and had outlined a series of potential steps that occur in the induction process. Since several of the initiating factors in addition to causing increased impulse flow to the organ, also induce the release of corticosterone from the adrenal, the direct effect of steroids on the induction process was studied. It was found that in the superior cervical sympathetic ganglia, dexamethasone caused an induction of tyrosine hydroxylase 48 hours after administration. This induction

could be prevented by treating the animals with the corticosterone receptor blocker, cortexalone. While this would suggest that a typical steroid induction was occurring, it was also found that activation of nicotinic receptors in the ganglion was required for the steroid induction. Comparable experiments with the adrenal medulla indicated that activation of steroid receptors in this tissue played no role in the transsynaptic induction of tyrosine hydroxylase.

The carotid body serves as a chemoreceptor organ with regard to blood gases. Dopamine containing cells as well as sympathetic nerve endings are found in the carotid body. A very sensitive assay has been developed to measure tyrosine hydroxylase in this tiny organ of the rat. Exposure of rats to hypoxic conditions results in a significant induction of tyrosine hydroxylase 24 hours after the stimulus. The induction required new protein synthesis and no changes in the kinetic properties of the induced enzyme were observed. The hypoxic conditions caused no change in tyrosine hydroxylase in the adrenal medulla or sympathetic ganglia. The induction in the carotid body was prevented by prior transsection of the carotid sinus nerve suggesting that this adaptive change was being mediated by a transsynaptic mechanism.

Serotonin-N-acetyltransferase (NAT). The regulation of NAT in the pineal gland provide a dramatic example of how nerve impulses can be translated into changes in enzyme activity of target cells. Activation of beta receptors cause up to a 50-fold increase in enzyme activity within 2 to 4 hours either in vivo or in organ culture. Work from other laboratories as well as our own was consistent with a mechanism that involved generation of cAMP, activation of protein kinase and subsequent activation of transcriptive events in the nucleus. Studies during the past year suggest that the apparent induction of NAT may not be entirely due to specific activation of transcription. We have found that no changes in total or specific types of RNA occurred following beta stimulation of cultured pineal glands. With the use of inhibitors that were relatively specific for different types of RNA synthesis it was possible to largely dissociate messenger RNA synthesis from the induction process. With the appropriate concentrations of cordycepin, messenger RNA synthesis could be inhibited 60 to 80% with very little effect on the increase in enzyme activity. Total inhibition of RNA synthesis by actinomycin D does however block the induction of NAT. It would appear that the message for NAT may be unusual; e.g., lacking poly A terminus, or that some form of translational control for NAT synthesis exists.

Sympathetic control of vascular protein synthesis. Previous studies from this laboratory had demonstrated that the spontaneously hypertensive rats incorporated lysine into non-collagen proteins of their mesenteric arteries at a much faster rate than the parent normotensive strain from Kyoto. This increased incorporation occurred even at an age (33 days) when their hypertensive disease was just beginning. During the past year we have found that this increased incorporation is apparently dependent upon intact sympathetic innervation since sympathectomy or sympathetic blockade markedly reduces lysine incorporation specifically in the small arteries. It does not appear to be a pressure related phenomenon since lowering the blood pressure with hydralazine does not decrease non-collagen synthesis. It may be that this represents another system in which nerve impulses control the synthesis of a specific protein in the target cell.

IV Catecholamine Metabolism in Genetic Models of Hypertension and in Man

Genetic animal models. A substrain of the spontaneously hypertensive rats (SHR) which has higher blood pressure and strong tendency to develop stroke has been introduced into the animal colonies of the NIH. These rats are called the stroke-prone SHR (SHR-SP). Examination of regional activity of tyrosine hydroxylase and DBH in the brain of SHR, SHR-SP, and Kyoto-Wistar rats revealed a small but significant increase in the activities of both these enzymes in the hypothalamus during the early development of hypertension. The activity of the two enzymes in the hypothalamus of the two hypertensive strains became similar to the control rats by 8 weeks of age and remained so thereafter. The activity of the hypothalamic enzymes was greater in the SHR-SP than the SHR, although the difference was not statistically significant. It is of interest that on an individual basis there was a significant correlation ($p < 0.001$) between the blood pressure increase and hypothalamic DBH from 5 to 8 weeks of age. In the periphery both the hypertensive strains had elevated serum DBH and norepinephrine at 3 weeks of age while at 6 weeks the only significant peripheral difference was an elevated plasma norepinephrine content in the SHR-SP. It would appear from these studies that neuronal factors may participate in the development of high blood pressure in these animals.

Catecholamine metabolism in essential hypertension in man. A comparison of 20 patients with essential hypertension with 20 age, sex and race matched normotensive subjects in regard to plasma DBH and norepinephrine has just been completed. In this study the above parameters of catecholamine metabolism were measured after 30 and 40 minutes of rest and after 2 and 10 minutes of standing in subjects whose blood pressure had been monitored for the previous 8 weeks. There was no significant difference in plasma DBH between the two groups and no significant rise in DBH occurred as a result of a 10 minute stand. Conversely, the hypertensive group had a mean value for plasma norepinephrine that was about 30% higher than the control group. The response to standing was the same in both groups each having a 100% rise in plasma norepinephrine following a 10 minute stand. These results indicate that hypertensive subjects may have slightly elevated sympathetic activity and that plasma DBH is not a good index of acute sympathetic activity.

Cerebrospinal fluid DBH as an index of central noradrenergic activity. Although there is some question as to whether serum DBH is an index of sympathetic nerve activity, it is possible that measurement of this enzyme in cerebrospinal fluid (CSF) might be an index of noradrenergic activity in brain. Early studies indicated that the amount of this enzyme in the CSF was too small to measure accurately. We have developed a more sensitive assay and can now accurately measure DBH in this fluid. In the past year we have examined about 50 normal and psychiatric patients. CSF activity is about 1/1000 that of serum and normals exhibit about a 10-fold range. While our experience in disease states is still very limited there was a tendency for DBH to be low in the CFS from schizophrenic patients. In 4 out of 5 patients with affective illness there was a marked increase in DBH of the CSF as the patient recovered from episodes of mania. These encouraging early results suggest that measurement of this enzyme may be an important tool in studying certain human diseases. The question of whether DBH could be an index of central noradrenergic remains unanswered.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01841-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Binding and Metabolic Effects of Neurotoxic Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald F. Bogdanski	Pharmacologist	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. on Biochem.Pharmacol.	HE NHLI
	Hans G. Baumgarten	Visiting Scientist	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The neurotoxic drugs 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine are currently in wide use in pharmacological laboratories because of their ability to destroy specific cells in the central nervous system. The cells destroyed belong to specific cell groups having a particular neurotransmitter. At present, the mechanism of action of the neurotoxic drugs is unknown. Evidence reported from this laboratory suggests that specificity is a matter of uptake by the cell. The drugs may be generally toxic to all cells provided they penetrate to the interior. The purpose of the present investigations was to determine the mechanism of toxicity and describe it at a molecular level if possible. It was thought that the toxicity might be related to the formation of quinones. If so, the site of a generalized toxicity might be mitochondria, in which the transport of electrons from NADH is inhibited by certain quinones, such as rotenone. This idea served as the starting point for the experiments to be described.

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Objectives: To help determine the mechanism of action of neurotoxic drugs.

Materials and Methods: Radioactive C^{14}-labeled 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine, and C^{14}-parachloroamphetamine were synthesized and supplied by Dr. K. Schlossberger of the Max Planck Institute, Germany.

The subcellular distribution of labeled drugs in brains of rat that had received intraventricular injections of these drugs was investigated by density gradient centrifugation. The 12,000 xg (P_2) pellet was resuspended and layered over a discontinuous density gradient consisting of 1.4, 1.2, 1.0, 0.8 M sucrose and the P_2 suspension in 0.32 M sucrose. After 90 minutes centrifugation at 56,000 xg, the particles fractionate at the various interphases, from low to high density, as follows: A, myelin; B, membranes and small synaptosomes; C, synaptosomes and small mitochondria; D, mitochondria and large synaptosomes; E, nonspecific debris.

The gradient is drained into counting vials, 5 drops per vial, and the radioactivity in each vial determined by scintillation counting.

Major Findings: This project, although promising, has been nearly inactive during the past year because of new commitments to other projects which were unforseen at this time last year. Four compounds which appear to cause specific toxicity with regard to serotonergic neuron are: 5,6-and 5,7-dihydroxytryptamine, p-chloroamphetamine and colchicine.

The different drugs used thus far can be classified according to their binding in specific cellular subfractions. Three distinct binding patterns were apparent: 1) both 5,6- and 5,7-dihydroxytryptamine were localized in the mitochondrial fraction. Moreover, 5,7-dihydroxytryptamine was apparently covalently bound as determined by exhaustive extraction with ether and methanol. Because of the tenacious binding, these compounds remained in the mitochondrial fraction for at least 24 hours, at which time the radioactivity in the other fractions had fallen to low levels. 2) Colchicine was present in higher concentrations in the synaptosomal fraction than in other fractions. The rate of disappearance at 1, 4, and 24 hours was proportional in all fractions. 3) Twenty-four hours after injection of parachloroamphetamine was found primarily in the membrane and the myelin fraction.

In contrast to the above compounds 5-hydroxytryptamine disappeared from all fractions within 24 hours. Thus, all neurotoxic drugs are tightly bound to specific cell fractions in brain tissue.

The data from experiments with the dihydroxytryptamines, together with earlier morphological data, suggest a mitochondrial site of action for these drugs. In preliminary experiments 5,7-dihydroxytryptamine and 6-hydroxydopamine inhibited reduction of cytochrome b in the presence of NADH and cyanide in rat liver and bovine adrenal medullary mitochondria.

Significance to Biomedical Research and Institute Program: Interest in these compounds is related to their ability to destroy specific groups of cells in the brain. For example, the tryptamines can eliminate serotonergic cells whereas 6-hydroxydopamine can eliminate adrenergic neurons. The specificity of neurotoxicity can thus be used to study the functions of a specific group of neurons in the central nervous system.

The mechanism of toxicity is currently the object of a lively controversy in biomedical research. The investigations reported here suggest that the dihydroxytryptamines destroy cells directly by their mitochondrial toxicity. The manufacture of vital energy resources, or other mitochondrial functions, might be blocked. This would cause the death of the cell. Specificity of cell toxicity is determined by the entrance of the compound into the cell. Serotonergic cells transport these compounds, hence they are destroyed.

Proposed Course of Project: The main objective of future research is to extend observations on mitochondrial toxicity. The inhibitory effect of neurotoxic drugs on electron transport, respiration, oxidative phosphorylation, lipid metabolism and protein metabolism will be studied. The possible usefulness of the dihydroxytryptamines in carcinoid tumors should be considered.

Publications:

1. Baumgarten, H.G., Bjorklund, A. and Bogdanski, D.F.: Similarities and differences in the mode of action of 6-hydroxydopamine and neurotoxic indoleamines. In Jonsson, G., Malmfors, T., Sachs, C. (Eds.): Chemical Tools in Catecholamine Research I. Amsterdam, North-Holland Publishing Co., 1975, pp. 59-66.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01843-03 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanisms of Transport: Uptake and Release of Biogenic Amines in Nerve Endings. (revised title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald F. Bogdanski Pharmacologist HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

This is part of a continuing project to study the role of electrolytes in the storage, uptake and release of biogenic amines in nerve endings. According to previous reports norepinephrine is thought to be mobilized by a calcium dependent mechanism that is regulated by intracellular sodium. The efflux is mediated by a sodium-facilitated mechanism that can be inhibited by desipramine, suggesting the involvement of transport. For the studies reported below, apparent outward transport of tritiated norepinephrine (^{3}H-NE) in sympathetic nerve endings was studied in rat heart slices incubated in a sodium-deficient medium (choline$^+$). Both efflux and uptake were inhibited by cocaine, desipramine and phenoxybenzamine. The ID_{50} for each drug is approximately equal for both effects. Extracellular NE exchanges with intracellular NE and the exchange is mediated by two mechanisms having apparent K_m values of 0.14 and 1.3 μM. Ouabain is a good inhibitor of uptake but it is a poor inhibitor of efflux. It is concluded that transport is mediated by one or more mechanisms and that the mechanisms for uptake and efflux are similar or identical. If so, ouabain inhibits transport indirectly, unlike the other inhibitors. These conclusions support the sodium-gradient hypothesis of transport.

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Objectives: To demonstrate the occurrence of carrier mediated outflow of norepinephrine in rat heart slices.

Major Findings: If efflux is mediated by a mechanism that is similar or identical to the carrier mediated uptake, then exchange transport should be demonstrable. In fact, exchange transport was demonstrated for extracellular NE in concentrations ranging from 0.01 to 30 μM. Transport was not limited to exchange, however. In the presence of 0.015 μM ℓ-NE, a very low concentration, there was a net uptake of 0.02 μmole ℓ-NE/0.5 g/15 min. Moreover, the concurrent efflux represented mainly spontaneously released NE. Net uptake relative to the stimulated release of NE was much higher although it was not possible to calculate because of the small quantities involved. Exchange was greatly reduced by cocaine, a transport inhibitor.

A double reciprocal plot of efflux against the concentration of extracellular norepinephrine suggested that more than one process was involved in efflux. Despite the inherent weakness of attempts to extract more than one kinetic constant from multiphasic Lineweaver-Bank plots, two values for the apparent K_m were about an order of magnitude apart suggesting that these values are tentatively acceptable, indeed one value of 0.14 μM is very similar to most reported values of the apparent K_m for uptake. The other was an order of magnitude higher. A few reports in the literature suggest that amines are transported by more than one mechanism in nerve endings, but the relationship between the phenomena described in the various reports is not clear.

Another method of demonstrating transport mediated efflux would be to inhibit the efflux with inhibitors of transport. We have previously reported that efflux is stimulated by incubating heart slices in Na^+-free media containing Ca^{++}, and that the stimulated efflux was inhibited by desipramine, an inhibitor of transport. The current evidence shows that the efflux was inhibited by cocaine and phenoxybenzamine as well. Moreover, all three drugs inhibited uptake and efflux. The ID_{50} concentrations were similar for both directions of translocation. For cocaine, a so-called pure inhibitor of uptake, the dose response curve for the effect representing a wide range of concentrations of cocaine on uptake was strikingly similar to the curve for the effect of cocaine on efflux.

In contrast, ouabain was a good inhibitor of uptake, but did not inhibit efflux at all. If uptake and efflux are, in fact, mediated by a similar mechanism, then the evidence indicates that ouabain does not directly inhibit the amine transporting mechanism. Ouabain also did not interfere with, or increase the efflux inhibiting effects of the transport inhibitors. Presumably, ouabain acts by eliminating electrolyte concentration gradients during uptake. Ouabain fails to inhibit efflux by this mechanism as the Na^+ gradient is already reversed and ouabain cannot raise the concentrations of intracellular Na^+ in the absence of extracellular Na^+.

Unlike synaptosomes in which uptake is almost abolished by ouabain, the drug does not inhibit uptake by heart slices by were than 60%. This evidence is

in accord with the proposed existence of more than one mechanism for transport of amines in adrenergic nerve endings.

Preliminary data (Report No. Z01 HL 01843-02 HE) indicating that phenoxybenzamine differed from the effects of cocaine and desipramine in a particular qualitative phenomenon were not confirmed.

Significance to Biomedical Research and Institute Program: Investigations of the physiological and biochemical characteristics of nerve endings generally are of great interest because synaptic transmission is crucial to either the initiation, control or maintenance (or all three) of all bodily functions, both physical and mental. Synaptic transmission is the point of attack for many drugs currently used for a wide variety of illnesses ranging from hypertension to mental disease. Fundamental knowledge of the nature of the biochemical processes controlling synaptic transmission is crucial to the understanding of the actions of existing drugs, and the development of drugs.

The findings reported above together with published reports of inhibition of synaptic transmission by desipramine and apparent inhibition of overflow by cocaine, suggest that synaptic transmission includes a component of outward transport of NE, which can be blocked by transport inhibitors.

Proposed Course of Project: This aspect of the studies of transport mediated release of neurotransmitters will be completed along the lines indicated above and then terminated.

Publications:

1. Bogdanski, D.F.: Mechanisms of transport for the uptake and release of biogenic amines in nerve endings. In Battistin, G., Lajtha, A. and Levi, R. (Eds.): Transport Phenomena in the Nervous System, Physiological and Pathological Aspects. Plenum Press, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER: Z01 HL 01844-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Receptors Participating in the Induction of Tyrosine Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ingeborg Hanbauer	Staff Fellow	HE NHLI
OTHER: E. Costa	Chief, Lab. of Preclin. Pharmacol.	LPP NIMH

COOPERATING UNITS (if any)

Laboratory of Preclinical Pharmacology, NIMH, Saint Elizabeth's Hospital, Washington, D.C.

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Nicotine induces tyrosine hydroxylase in superior cervical ganglion. This action is facilitated by blockade and inhibited by activation of muscarinic receptors. The antagonistic effect of muscarinic agonists on the increase of TH activity elicited by nicotinic receptor agonists appears to be mediated by dopamine presumably stored in the SIF cells. In addition, dopamine receptor agonists prevent the induction of TH by nicotine, whereas prior depletion of dopamine stores by reserpine facilitates the action of nicotine on TH induction in decentralized ganglia.

PHS-6040
(12-75)

Objectives: In superior cervical ganglia an increased impulse traffic in the preganglionic nerve fibers causes an increase of tyrosine hydroxylase (TH) activity about 48 hours after the stimulation. While in adrenal medulla the stimulation of nicotinic receptors increases the cAMP content which activates a cytosol histone kinase associated with the induction of TH, in ganglia TH induction is not always preceded by an increase in cAMP content. This project was carried out to define the role of nicotinic and muscarinic receptors in the induction of TH in intact and decentralized superior cervical ganglia.

Methods: cAMP and cGMP concentrations were estimated by measuring the incorporation of ^{32}P into histones using cAMP- or cGMP-dependent protein kinases. TH activity was measured by a radiochemical technique using 6-methyltetrahydropterin as cofactor.

Major Findings: The use of pharmacologic agonists and antagonists made it possible to obtain a better understanding of the participation of cholinergic and dopaminergic receptors in the regulation of TH activity in superior cervical ganglia. Unlike the adrenal medulla, stimulation of nicotinic receptors failed to elicit an initial increase in cAMP content in superior cervical ganglia. While nicotine was equally active in inducing TH in intact and denervated adrenal medullae, it induced TH only in intact but not decentralized superior cervical ganglia. Experiments in which atropine and nicotine were administered together demonstrated that atropine lowers the nicotine threshold to induce TH in intact ganglia and facilitated the induction of TH by nicotine in decentralized ganglia. On the other hand, administration of methacholine a muscarinic receptor agonist antagonized the induction of TH by nicotine or reserpine. In superior cervical ganglia the endogenously stored catecholamines in SIF cells, which have been shown to be released by muscarinic receptor agonists, are involved in the blockade of TH induction by methacholine. In order to prove the participation of catecholamines in this inhibitory action, we studied the effect of apomorphine on TH induction elicited by nicotine or reserpine. Apomorphine inhibits TH induction by both agents. Furthermore, prior depletion of catecholamines by reserpine facilitates the induction of TH in decentralized ganglia by a subthreshold dose of nicotine.

Proposed Course of Project: In order to understand the molecular mechanism of TH regulation by muscarinic, nicotinic and dopaminergic receptors, more detailed studies on the effect of the various receptor agonists and antagonists on the activation of protein kinase, phospho-protein phosphatases and RNA-synthesis in superior cervical ganglia will be required.

Publications:

1. Hanbauer, I. and Costa, E.: Induction of tyrosine hydroxylase in the superior cervical ganglia of rats: Opposite influence of muscarinic and nicotinic receptor agonists. Neuropharmacology 15: 85-90, 1976.

2. Hanbauer, I. and Costa, E.: Trans-synaptic induction of tyrosine hydroxylase in superior cervical ganglia: Participation of post-synaptic and steroid receptors. In Almgren, O., Carlsson, A. and Engel, J. (Eds.): Chemical Tools in Catecholamine Research Vol II. North Holland Publ. Co. 1975, pp. 175-182.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01845-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Tyrosine Hydroxylase by Steroid Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Staff Fellow	HE NHLI
OTHER:	E. Costa	Chief, Lab. of Preclin. Pharmacol.	LPP NIMH
	A. Guidotti	Chief, Sect. of Neuroendocrinology	LPP NIMH
	W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)

Laboratory of Preclinical Pharmacology, NIMH, Saint Elizabeth's Hospital, Washington, D.C.

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Carbohydrate active steroids facilitate the transsynaptic induction of tyrosine hydroxylase (TH) in superior cervical ganglion. The increase in ganglionic TH activity did not occur in rats with impaired adrenal cortical function and after administration of cortexolone- a specific corticosterone receptor antagonist.

PHS-6040
(12-75)

Objectives: In various tissues enzyme induction is controlled by steroid hormones. It is presently accepted that responses to carbohydrate active steroids are mediated by specific steroid receptor molecules in the cytoplasm. The steroid-receptor protein complex is translocated to the nucleus where subsequent interaction with repressor proteins presumably alters transcriptional processes. They appear to initiate a sequence of nuclear events, including RNA, DNA and specific protein synthesis.

Methods: cAMP concentration was measured by ^{32}P-incorporation into histones catalyzed by a cAMP dependent protein kinase. TH activity was measured by a radiochemical technique with 6-methyltetrahydropterin as cofactor.

Major Findings: Earlier experiments with adrenal demedullated rats subjected to cold stress suggested that corticosterone receptors play a role in the regulation of ganglionic TH. These results stimulated our interest in studying the action of carbohydrate active steroids on the regulation of TH synthesis. In superior cervical ganglia but not in adrenal medulla dexamethasone increases TH activity about 48 hours after injection. This increase can be antagonized with cortexolon - a specific blocker of corticosterone receptors. The action of dexamethasone requires intact innervation of the ganglion and fails to occur in rats, which were decentralized three days before the experiment. Since reserpine elicits an increase of plasma corticosterone level lasting at least 6 hours, we studied a possible involvement of carbohydrate active steroids in the trans-synaptic induction of TH. Our experiments showed that in rats injected with reserpine the induction of ganglionic TH depends upon activation of nicotinic receptors in addition to an activation of corticosterone receptors since it could be antagonized by cortexolone.

Significance to Biomedical Research and Institute Program: The interaction of carbohydrate active steroids with catecholaminergic neurons appears to be an important mechanism whereby neurons change their macromolecular composition to adapt to persistent environmental changes.

Propose Course of Project: Our future studies on the involvement of steroid receptors in TH induction will include studies on 1) regulation of phosphorylated state of histone and nonhistone nuclear proteins and 2) the regulation of cytoplasmic and membrane bound phospho-protein phosphatases.

Publications:

1. Hanbauer, I., Lovenberg, W., Guidotti, A., and Costa, E.: Role of cholinergic and glucocorticosteroid receptors in the tyrosine hydroxylase induction elicited by reserpine in superior cervical ganglion. Brain Res. 96: 197-200, 1975.

2 Hanbauer, I. and Costa, E.: Trans-synaptic induction of tyrosine hydroxylase in superior cervical ganglia: Participation of post-synaptic and steroid receptors. In Almgren, O., Carlsson, A. and Engel, J. (Eds.): Chemical Tools in Catecholamine Research Vol II. North Holland Publ. Co. 1975, pp. 175-182.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01846-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Tyrosine Hydroxylase in Carotid Body

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbäuer	Staff Fellow	HE NHLI
OTHER:	E. Costa	Chief, Lab. of Preclin. Pharmacol.	LPP NIMH
	W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI
	S. Hellstrom	Assistant Professor	

COOPERATING UNITS (if any)
Laboratory of Preclinical Pharmacology, NIMH, Saint Elizabeth's Hospital, Washington, D.C.
Deparment of Anatomy, University of Umea, Umea, Sweden

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the mechanism of tyrosine hydroxylase (TH) induction in glomus cells of the carotid body. Exposure to hypoxic conditions causes an initial selective decrease in the dopamine concentration and a long-term increase in TH activity. Since the increase in TH activity can be abolished by cycloheximide increased protein synthesis appears to be involved. Blockade of TH-induction by trans-section of the carotid sinus nerve proposes the existence of a reciprocal synapse between glomus cell and sinus nerve terminal.

PHS-6040
(12-75)

Objectives: The carotid body senses physiologic oscillation of arterial pO_2, pCO_2 and $[H^+]$. In the carotid body of serveral animal species dopamine and norepinephrine are contained in glomus cells which are proximal to the terminals of the carotid sinus nerve. It has been suggested that the glomus cell may participate in the chemoreceptive function of the carotid body either directly or as modulator of the firing rate of the impinging sinus nerve terminals. Our research goal is to study the molecular mechanisms for the regulation of TH during hypoxia.

Methods: For measurements of blood gases one carotid artery of rats was cannulated and blood was drawn before, during and after hypoxia and analyzed in a blood gas analyzer. TH activity was measured with a radiochemical assay with 6-methyltetrahydropterin as cofactor. Activation of RNA synthesis was determined by the incorporation of ^3H-uridine with and without α-aminitin to selectively block RNA polymerase II. Phosphoprotein phosphatase activity was measured by assaying the ^{32}P-orthophosphate released from phosphorylated histones.

Major Findings: Exposure to hypoxic conditions (5% O_2 and 95% N_2) lowered the pO_2 significantly and the pCO_2 slightly but failed to change arterial $[H^+]$. Exposure to hypoxia causes a selective decrease of the dopamine content whereas norepinephrine content remained unchanged. Sufficient exposure to hypoxic conditions elicited a two-fold increase of the V_{max} for tyrosine hydroxylase, but the K_m for the pterin cofactor remained unchanged. Since the increase in TH activity is abolished by cycloheximide treatment an increased synthesis of new enzyme molecules appears to be involved. Furthermore, monolateral transsection of the carotid sinus nerve abolished the long-term increase of TH activity in carotid body but did not prevent the reduction of the dopamine concentration. These results imply that the release of dopamine is not restricted to the synaptic contacts with nerve fibers and probably can not be entirely assocaited with the chemoreceptor function. Moreover the increase in TH activity depends on intact chemoreceptive nerve terminals. The increase in TH activity elicited by hypoxia is not abolished by blockers of muscarinic or nicotinic receptors implying that stimulation of these receptors is not involved in the response of TH activity to this specific stimulus.

In order to reveal some of the molecular mechanisms preceding the induction of TH by hypoxia studies on phosphodiesterase, phosphoprotein-phosphatase and RNA-synthesis were performed. One hour after exposure to hypoxic conditions the high K_m form for cAMP of phosphodiesterase is shifted to a low K_m form indicating that cAMP may be the second messenger involved in this regulation. Furthermore, exposure to hypoxic conditions elicits an activation of phosphoprotein phosphatases and an increased incorporation of uridine into RNA.

Significance to Biomedical Research and Institute Program: From our studies it can be inferred that chemosensitivity is a specific property of carotid sinus nerve endings impinging on carotid glomus cells. Since stimulation of the carotid sinus nerve causes a release of dopamine which in turn was

shown to abolish the discharge of the sinus nerve the existence of a reciprocal synapse between glomus cell and sinus nerve terminal can be proposed. The importance of these reciprocal synapses appears to lie in the response to acute hypoxic conditions as well as adaptation to environmental hypoxia in high altitude or deep sea diving.

Proposed Course of Project: The following experimental approach will be undertaken to further reveal the mechanisms of TH induction elicited by hypoxia:

1. Pharmacologic studies on the release of first messenger involving mass fragmentographic determination of dopamine and norepinephrine content.

2. Studies on the involvement of second messengers, which will involve radioimmunoassay of cAMP as well as in vitro formation of this nucleotide through activation of adenylate cyclase.

3. Studies on cyclic-nucleotide dependent and independent protein kinases.

Publications:

1. Hanbauer, I.: Mechanisms for tyrosine hydroxylase induction in sympathetic ganglia and carotid body. In Costa, E., Giacobim, E. and Paoletti, R. (Eds.): Advances in Biochemical Psychopharmacology Vol 15. Raven Press, N.Y., 1976, in press.

2. Hanbauer, I. and Hellstrom, S.: Selective induction of tyrosine hydroxylase in carotid body of rats exposed to hypoxic conditions: Evidence for a participation of dopaminergic reciprocal synapses. Fed. Proc. 35:(3) 1540, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01847-02 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Role of Second Messengers in Tyrosine Hydroxylase Induction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Staff Fellow	HE NHLI
OTHER:	E. Costa	Chief, Lab. of Preclin. Pharmacol.	LPP NIMH
	A. Guidotti	Chief, Sect. of Neuroendocrinology	LPP NIMH

COOPERATING UNITS (if any)

Laboratory of Preclinical Pharmacology, NIMH, Saint Elizabeth's Hospital, Washington, D.C.

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

Stimulation of adrenal chromaffin cells results in an increase in cAMP/cGMP ratio, which initiates a cascade of biochemical events leading to a delayed increase of TH activity. In superior cervical ganglia a correlation between increase of cAMP content and TH-induction exists after stimulation of β-adrenergic receptor and cold exposure of adrenal demedullated rats.

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PHS-6040
(12-75)

Objectives: The goal of these studies is to understand the involvement of first and second messengers in the control of tyrosine hydroxylase (TH) induction in adrenal medulla and sympathetic ganglia. In adrenal medulla direct or indirect stimulation of nicotinic receptors elicits an increase in TH activity, which is preceded by an increase in cAMP content. We applied this model for studies on sympathetic ganglia, which in their synaptic organization appear more complex than adrenal chromaffin cells.

Methods: cAMP and cGMP concentrations were estimated by measuring the incorporation of ^{32}P into histones by cAMP- or cGMP-dependent protein kinases. TH activity was measured by a radiochemical technique using 6-methyltetrahydropterin as cofactor.

Major Findings: TH induction caused by nicotinic receptors stimulation of adrenal chromaffin cells is proceeded by a stimulus coupled increase of cAMP concentration and a decrease in cGMP content lasting for more than one hour. We observed that in adrenal medulla any stimulus eliciting an increase in cAMP/cGMP ratio lasting less than one hour does not promote TH-induction. The regulation of TH activity in superior cervical ganglia appears to involve a variety of mechanisms and only under specific conditions is the increase in cAMP content the necessary initial event promoting the induction of TH. In intact and decentralized superior cervical ganglia stimulation of β-adrenergic receptors lasting for 2 hours causes a sustained increase of ganglionic cAMP content and TH-induction. Both changes can be prevented by administration of β-receptor antagonists. Furthermore, in adrenal demedullated rats, exposure to 4° for 4-6 hours elicits an early increase in cAMP/cGMP ratio followed by a delayed increase in TH activity. Since intact preganglionic innervation is required to obtain the increase in both parameters, a trans-synaptic induction mechanism via cholinergic synapses is proposed. In fact, up to 4 hours after onset of the stimulation ganglionic blocking agents prevent both increase in cAMP content and TH activity, whereas at a later time this increase is no longer impaired, suggesting that the elaboration of the stimulus is independent from ganglionic transmission.

Proposed Course of Project: The goal of this project is to elucidate the role of cAMP and cGMP in activation of proteinkinases which initiate the phosphorylation of non-histone nuclear proteins. In order to succeed in this project we intend to develop a sensitive radioimmunologic determination of cAMP and cGMP in the femtomole range.

Publications:

1. Hanbauer, I., Guidotti, A. and Costa, E.: Involvement of cyclic nucleotides in the long term induction of tyrosine hydroxylase. In Neuropsychopharmacology, Proc. of IX Congress of CINP, Excerpta Medica, Amsterdam, 1975, pp. 932-941.

2. Costa, E., Guidotti, A. and Hanbauer, I.: Comment on the paper "Lack of Correlation between rate of Increase in cAMP and subsequent induction of tyrosine hydroxylase in sympathetic ganglia and adrenal medulla by H. Thoenen, U. Otten, R.A. Mueller, R. Goodman and F. Oesch. In Neuropsychopharmacology, Proc. of IX Congress of CINP, Excerpta Medica, Amsterdam, 1975, pp. 952-955.

3. Costa, E., Guidotti, A. and Hanbauer, I.: Cyclic nucleotides and trophism of secretory cells: Study of adrenal medulla. In Weiss, B. (Ed.): Cyclic Nucleotides in Disease, Philadelphia, 1975, pp. 167-186.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01848-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Dopamine-β-hydroxylase in Human Cerebrospinal Fluid
(revised title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pauline Lerner	Postdoctorate Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI
	Frederick Goodwin	Chief, Sect. on Psychiatry	LCS NIMH
	Gerald Brown	Medical Officer	AP NIMH

COOPERATING UNITS (if any)
Adult Psychiatry Branch, NIMH; National Naval Medical Center, Bethesda, Md.; Laboratory of Clinical Science, NIMH

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

SUMMARY OF WORK (200 words or less - underline keywords)

Dopamine-β-hydroxylase (DBH) is the enzyme which catalyzes the conversion of dopamine to norepinephrine in noradrenergic neurons. We are measuring DBH in human cerebrospinal fluid, and the amount of this enzyme is taken as an index of central noradrenergic activity. Subjects are psychiatric patients who have been hospitalized for schizophrenia, affective illness, alcoholism, or personality disorders. Preliminary data indicate that DBH tends to be low in CSF from schizophrenic patients, and that DBH levels in cerebrospinal fluid from patients with affective illness are related to the clinical symptoms of the disease.

794

PHS-6040
(12-75)

Objectives: We are interested in studying the activity of dopamine-β-hydroxylase (DBH) in the human central nervous system in patients with psychiatric disorders. DBH is found in noradrenergic neurons, and is released from its neuronal storage sites, along with norepinephrine, when the neurons fire. For these reasons, we feel that DBH activity in cerebrospinal fluid (CSF) would be a useful indicator of central noradrenergic activity. It has been suggested, on the basis of DBH assays of human brains at autopsy, that brain DBH is low in schizophrenics. Two different groups have reported conflicting results on this point, and it is possible that technical difficulties arising from the instability of post mortem samples are responsible for the discrepancy. We have, therefore, undertaken to measure DBH in CSF from psychiatric patients and to try to correlate DBH activity with clinical symptoms and drug treatment.

Methods: Cerebrospinal fluid is taken from psychiatric patients who have been hospitalized for schizophrenia, affective illness, alcoholism, or personality disorders. Because the concentration of DBH in CSF is extremely low, a very sensitive assay is required. Our initial attempts to detect DBH in CSF with a radioimmunoassay were unsuccessful. We are now using a radioenzymatic method to measure DBH in CSF. We have modified a previously existing assay method by changing the incubation conditions and by using ^{14}C labeled substrate of very high specific activity. This assay is sensitive enough to reproducibly detect and quantitate the very small amount of DBH present in CSF.

Major Findings: We have analyzed approximately fifty CSF samples thus far. The DBH levels cover a ten-fold range, and dramatic differences are apparent among the subjects studied. There is a tendency for DBH activity to be low in samples from schizophrenics. Lumbar punctures have been obtained from several patients at different times, so that DBH levels can be related to symptoms and treatment. Preliminary data indicate that there are consistent changes in DBH levels in CSF of patients with affective illness who are experiencing or recovering from manic or depressive episodes. Although our results tend to support the involvement of the central noradrenergic system in schizophrenia and affective illness, more extensive studies are needed to substantiate and clarify the relationship.

Significance to Biomedical Research and Institute Program: The measurement of DBH activity in CSF appears to be a useful technique for obtaining information on central noradrenergic activity in human subjects. Analysis of DBH in CSF of psychiatric patients should help to elucidate the role of brain norepinephrine in mental illness.

Proposed Course of Project: We plan to expand our study to include larger numbers of patients, since the validity of our conclusions will depend, in part, on the number of subjects studied. We also intend to investigate the effects of drug treatment on central noradrenergic activity, as expressed by DBH in CSF.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01849-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Synthesis of Dopamine-β-hydroxylase in a Cell Free System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pauline Lerner	Postdoctorate Fellow	HE NHLI
	Paul MacDonnell	Postdoctorate Fellow	LBS NICHHD
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI
	Gordon Guroff	Chief, Sect. Intermediary Metabolism	LBS NICHHD

COOPERATING UNITS (if any)

Section on Intermediary Metabolism, Laboratory of Biomedical Sciences, National Institute of Child Health and Human Development, Bethesda

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Dopamine-β-hydroxylase (DBH) is an enzyme which is required for the biosynthesis of norepinephrine and epinephrine. It is known that neuronal activity can influence the rate of synthesis of DBH by transsynaptic induction. We are attempting to devise a cell free system in which the synthesis of DBH can be investigated. We are using RNA from bovine adrenal medulla and rat superior cervical ganglion as templates for protein synthesis. A sensitive and specific radioimmunoassay has been developed for the detection of the DBH.

PHS-6040
(12-75)

Objectives: Dopamine-β-hydroxylase (DBH) is the enzyme which catalyzes the final step in the biosynthesis of norepinephrine. It is found in the adrenal medulla and in noradrenergic neurons. It is known that the synthesis of DBH is regulated, in part, by neuronal factors. However, the molecular mechanism by which neuronal activity affects protein synthesis is not well understood. The objective of this project is to devise a cell free system in which DBH synthesis can be studied. This involves the development of an appropriate *in vitro* system for protein synthesis and the detection and quantitation of the DBH synthesized.

Methods: Fresh bovine adrenal glands are used to prepare RNA. Either polysomes or polyadenylic acid (poly A) RNA is used as the template for protein synthesis. The other factors required for protein synthesis are obtained from a preparation of untoasted wheat germ.

The protein products of the cell free synthesis are assayed for DBH by means of a radioimmunoassay. A solid phase method of radioimmunoassay is employed, using an antiserum raised against purified bovine DBH. The antibody is bound to very small (0.5 micron in diameter) polystyrene beads, which, in turn, are allowed to bind to DBH.

Major Findings: At first, we used the messenger RNA fraction (poly A RNA) from bovine adrenal medulla as the template for protein synthesis. The poly A RNA stimulated protein synthesis to a level four to seven times as high as the background from the wheat germ system alone. Using our radioimmunoassay, we were able to detect the DBH which was produced. However, evidence from ultracentrifugation and gel electrophoretic studies indicated that incomplete DBH molecules were being made. Recently, we have changed our procedure so that polysomes from bovine adrenal medulla serve as the source of RNA. With this method, protein synthesis is increased several fold, and larger proteins are made. We were able to detect DBH among the products of protein synthesis in some experiments, but our results have not been consistent. We are now trying to modify the conditions of the protein synthesis and the radioimmunoassay so that DBH can be reproducibly synthesized and detected.

Significance to Biomedical Research and Institute Program: DBH is necessary for the *in vivo* synthesis of the catecholamines norepinephrine and epinephrine. The catecholamines are of particular interest because of their role in the modulation of blood pressure. The regulation of DBH synthesis is one of several mechanisms by which neuronal activity can affect blood pressure.

Proposed Course of Project: We are now trying to devise a cell free system which will reproducibly synthesize DBH. If we are successful in detecting newly synthesized DBH with our immunoassay, we also intend to further characterize the putative DBH by chromatographic techniques. One possibility which we are investigating is the optimization of the system which utilizes bovine adrenal medullary RNA. Another possibility is the use of RNA from superior cervical ganglia maintained in organ culture. The latter system is attractive because it could be readily used to study the effects of pharmacological agents on DBH synthesis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01850-07 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Biochemistry of the Spontaneously Hypertensive Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Akinobu Nagaoka	Visiting Scientist	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

SUMMARY OF WORK (200 words or less - underline keywords)

The brain regional activities of tyrosine hydroxylase, dopamine-β-hydroxylase and tryptophan hydroxylase have been measured as a function of age in Kyoto-Wistar rats (WK), spontaneously hypertensive rats (SHR) and stroke-prone rats (SHR-SP). Small changes in enzyme activity were noted during the developmental phase of the hypertension. Perhaps the most significant change was an increase in tyrosine hydroxylase and dopamine-β-hydroxylase in the hypothalamus during weeks 5-8. On an individual basis there was a highly significant correlation between hypothalamic dopamine-β-hydroxylase and the increase in blood pressure during this period. In the periphery both plasma norepinephrine and plasma dopamine-β-hydroxylase were elevated at 3 weeks of age suggesting an early involvement of the sympathetic nervous system in the development of hypertension.

PHS-6040
(12-75)

Objectives: Extensive evidence from studies on metabolism of biogenic amines or activity of the related enzymes indicates that catecholaminergic and serotonergic nerves may play an important role in the development of experimental hypertension. Although we have also studied the central neural mechanisms in the spontaneously hypertensive rats (SHR), the details (nature and extent) of their participation has not yet been established. Recently, a stroke-prone strain of the SHR was introduced to NIH from Japan. The stroke-prone SHR have much more rapid and greater elevation of blood pressure as compared with the regular SHR, which have been used as a model of human essential hypertension for the last decade. The objective of the current experiments is to reexamine some of the neural factors that may participate in the development of hypertension by comparing the stroke-prone SHR, regular SHR and the control Wistar-Kyoto rats (WKR).

Methods: Male SHR, stroke-prone SHR and WKR used in these studies were obtained from the Animal Production Section of the NIH. For comparative purposes, two types of experimental hypertension were produced in male WKR. One of them was mineralocorticoid (DOCA) and salt hypertension and the other was renal hypertension.

The activities of monoamine biosynthetic enzymes were measured in brain regions of these kinds of hypertensive rats at various ages. Tyrosine hydroxylase and dopamine-β-hydroxylase (DBH) activities were assayed using sensitive radio-enzymic methods. Tryptophan hydroxylase was measured by a spectrofluorometric method. In the periphery, plasma DBH activity and norepinephrine were examined. Systolic blood pressure was measured the day before the sacrifice by a tail-cuff method.

The statistical significance was determined by an unpaired Student's t-test, and a probability of less than 0.05 was considered significant.

Major Findings: The SHRs had significantly elevated blood pressures as compared to the control WKR after 5 weeks of age (the blood pressure levels: stroke-prone SHR > regular SHR > WKR). During the early development of hypertension in the SHRs, the activity of tyrosine hydroxylase in the hypothalamus and corpus striatum and of dopamine-β-hydroxylase in the hypothalamus and pons medulla were significantly higher than in the controls. The changes of these catecholamine synthesizing enzymes, especially in the hypothalamus, significantly correlated with the level of blood pressure. In contrast, the activities of tyrosine and dopamine-β-hydroxylase were slightly decreased in the DOCA-salt and renal hypertensive rats. Furthermore, the SHRs showed a significantly higher level of plasma DBH and norepinephrine (plasma DBH and norepinephrine levels: stroke-prone SHR > regular SHR > WKR). The increased levels were not observed in experimental hypertensive rats.

Significance to Biomedical Research and Institute Program: From increased activities of tyrosine hydroxylase and DBH in the hypothalamus and their correlation with the blood pressure, it was suggested that catecholaminergic neurons in the hypothalamus may participate in the initiation of high blood

pressure in the genetically mediated but not in the experimentally mediated hypertension. This finding was supported by the facts of increased DBH activity and norepinephrine level in plasma.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01851-02 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Tyrosine Hydroxylase in the Central Nervous System
(revised title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Matthew M. Ames	Research Associate	HE NHLI
OTHER:	Pauline Lerner	Postdoctorate Fellow	HE NHLI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term goal of this work is the understanding of the regulation of tyrosine hydroxylase in the central nervous system. Towards this end, we have studied the activation of the enzyme (via a decrease in cofactor K_m with phosphorylation and G-75 chromatography as well as the reported activation with EGTA and calcium. We have been unable to verify these reports. We have further found that there appears to be a relationship between activation via phosphorylation and catecholamine concentration. Future studies will focus on G-75 activation, dopamine Ki values under several conditions, and experiments designed towards the elucidation of a mechanism of activation common to all of the activating procedures.

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PHS-6040
(12-75)

Objectives: Tyrosine hydroxylase is the rate-limiting enzyme in the production of catecholamines in the central nervous system, and consequently, is intimately involved in the regulation of neurons which utilize catecholamine neurotransmitters. Several laboratories, including this one, have reported the activation of tyrosine hydroxylase by a variety of procedures. This short-term activation has been accomplished by additions to *in vitro* preparations (EGTA, calcium ion, phosphorylation conditions, phosphatidyl serine, heparin sulfate, etc.), treatment of animals with neuroleptic agents, nigro-striatal lesions, and Sephadex G-25 chromatography. The objectives of these studies have been to examine several such procedures which may be involved in the physiological regulation of tyrosine hydroxylase (phosphorylation, catecholamine concentration, calcium), and to examine kinetic properties of the enzyme in several brain regions in response to several of the above conditions. In this way, we hope to more fully elucidate the molecular events associated with these activations, and to further study reported differences in the properties of the enzyme in different brain regions.

Methods: The striata and hypothalamus of the brain and adrenals of male Sprague-Dawley rats were used as a source of tyrosine hydroxylase. The enzyme was assayed by the standard tritium release assay. The enzyme was assayed with either 2-mercaptoethanol or sheep liver reductase as the reducing system under a variety of conditions.

Major Findings:

Regulation of tyrosine hydroxylase by phosphorylation and catecholamine concentration. Recent work in this laboratory has demonstrated the activation of rat striatal tyrosine hydroxylase (decrease in apparent K_m for 6-methyltetrahydropterin from 0.5 mM to 0.15 mM) by addition of phosphorylation components to a 40,000 x g supernatant. It was also shown that this activation was retained after passing the mixture over Sephadex G-25 columns. Further preliminary experiments demonstrated that a similar activation could be obtained by passing a crude extract over such a column.

We have now found that passage of crude striatal or adrenal extracts over a Sephadex G-25 column of appropriate length to insure removal of most of the catecholamines (determined by studies employing dyes and dopamine) activate tyrosine hydroxylase. In such studies, the apparent K_m for the cofactor was decreased about three fold (0.5 mM to 0.15 mM). This decrease was identical to that obtained upon phosphorylation of crude extracts of adrenal or striatal tissue. Addition of dopamine (3×10^{-6}M) restored the enzyme to its control K_m form, further implicating catecholamines in this mode of activation.

We next explored the possible relationships between activation via phosphorylation and catecholamine concentrations in the tissues. In catecholamine rich tissues (striata and adrenals), it was found that phosphorylation did not further activate enzyme preparations previously passed over G-25 columns. While dopamine restored control K_m values to preparations passed over the G-25 columns, it did not restore that control to preparations passed over the G-25 columns and then phosphorylated. In the catecholamine poor hypothalamus,

we found that G-25 treatment did not further activate the enzyme, and that the crude enzyme itself had a lower K_m than in dopaminergic regions (0.2 mM vs. 0.5 mM). In this tissue we further observed that phosphorylation of the G-25 treated extract resulted in an activation beyond that of the G-25 treatment. These studies suggest that the regulation of tyrosine hydroxylase by phosphorylation may be dependent upon the level of catecholamines in the particular tissue or brain region.

The role of EGTA and calcium in tyrosine hydroxylase regulation in dopaminergic and noradrenergic brain regions. It has been reported by other workers that in dopaminergic brain regions addition of EGTA to in vitro preparations activates tyrosine hydroxylase, and that this activation may be prevented by calcium. Conversely, it has been reported that in noradrenergic regions, calcium activates tyrosine hydroxylase in similar preparations. It was also reported that in the dopaminergic regions, the K_I for dopamine was dramatically increased upon treatment with EGTA. We investigated these findings, utilizing both chemical and enzymatic systems for preservation of the cofactor in the reduced state. We have found no significant effect of either EGTA or calcium in noradrenergic or dopaminergic brain regions, and no change in the K_I for dopamine. We investigated these effects both at optimal and suboptimal conditions with respect to substrate and cofactor, as well as with both of the commonly used reduction systems (mercaptoethanol and sheep liver reductase). Our findings suggest that there is no major difference in tyrosine hydroxylase in different brain regions, but that the micro environment may be the key in observations showing kinetic difference in tyrosine hydroxylase in various brain regions.

Significance to Biomedical Research and Institute Program: It has previously been shown that the concentration of the natural tyrosine hydroxylase cofactor is suboptimal, and that the enzyme is probably operating at about 1% efficiency. Since the factors discussed above alter the enzyme activity by affecting the affinity of the enzyme for the cofactor, they may be of physiological importance. This is especially true for those present in the normal system, such as catecholamine concentration and phosphorylation. Thus, the investigation of these mechanisms may result in better understanding of neuronal regulation. Such an understanding may be of significance in disease states with central nervous system involvement.

Proposed Course of Project: We plan to further investigate the significance of phosphorylation as it relates to the concentration of catecholamines in several brain regions. We also will be examining the behaviour of the enzyme in dopaminergic and noradrenergic brain regions, to better understand the factors regulating the enzyme in these regions.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01852-05 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulation of Hydroxyindole Pathway in the Pineal Gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Morrissey	Staff Fellow	HE NHLI
OTHER:	W. Lovenberg	Chief, Sect. Biochem. Pharm.	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

SUMMARY OF WORK (200 words or less - underline keywords)

The scope of this project is to define the molecular events involved in the neuronal induction of serotonin-N-acetyltransferase(NAT) in the pineal gland. During the course of this investigation it was found that there seem to be no significant changes in RNA synthesis, protein synthesis or phospho-protein synthesis in cultured pineals during NAT induction by the β agonist isoproterenol. Protein kinase activity is markedly enhanced during the initial stages of isoproterenol induction prior to the rise in NAT activity.

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PHS-6040
(12-75)

Objectives: The hydroxyindole pathway of the pineal gland offers a unique opportunity to examine mammalian enzyme regulation and regulation of protein synthesis. The synthesis of melatonin is controlled by the levels of serotonin-N-acetyltransferase (NAT) which in turn is under neuronal control. Beta receptor stimulation causes a 50-fold increase in NAT activity. Cyclic AMP is the second messenger in this system. The objective of this project is to define at a molecular level the mechanism by which cAMP is eliciting the large increase in NAT activity.

Methods: The control of serotonin-N-acetyltransferase (NAT) was studied in the cultured rat pineal. Levels of the enzyme were measured in individual glands by a sensitive radiochemical technique. RNA synthesis was assayed by the incorporation of ^{3}H-uridine into cold TCA precipitable material in post-mitochondrial supernatants of pineal homogenates. The radiolabeled RNA was further characterized by phenol extraction and agarose gel electrophoresis. Various inhibitors of RNA synthesis were used to study the effect on the induction of NAT.

Protein synthesis in the cultured pineals was measured by the incorporation of ^{3}H or ^{14}C-labeled amino acids or ^{35}S-methionine into hot TCA precipitable proteins of post-mitochondrial supernatants. The radiolabeled protein products where characterized by SDS (sodium dodecyl sulfate) disc gel or SDS-slab gel electrophoresis and liquid scintilation counting or autoradiography. Phosphoprotein synthesis was assayed by the incorporation of ^{33}P-phosphate into cultured pineal glands. The post-mitochondrial supernatants were separated by SDS-slab gel electrophoresis and autoradiographed.

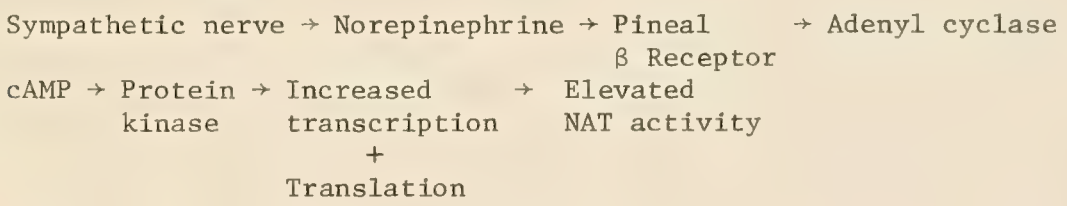
Protein kinase activity was also measured in cultured pineal glands at various times during the isoproterenol stimulated induction of NAT activity. The kinase was measured by the incorporation of ^{32}P into histone Type II-A using α-^{32}P-ATP and millipore filtration.

Major Findings: Maximum induction of NAT in cultured pineals is achieved 6 hours after the addition of the β-agonist isoproterenol. During the first 3 hours of induction there is no marked increase in enzyme activity while during the last 3 hours enzyme activity is elevated at least 30-fold. The initial phase of induction is sensitive to the RNA synthesis inhibitor actinomycin D, however, after 3 hours the process is insensitive to the inhibitor. This suggests that RNA synthesis is involved. The whole induction process is relatively insensitive to the inhibitor cordycepin (3-deosyadenosine, inhibits poly A addition) which suggest that either RNA synthesis is not involved or that a messenger RNA lacking poly A is produced and required for enzyme induction. The RNA synthesis inhibitor α Amanitin (inhibits mRNA synthesis directly and rRNA synthesis secondarily) does inhibit NAT induction in conjunction with inhibiting RNA synthesis. These findings taken together indicate that RNA synthesis is involved in the induction of pineal NAT activity and that the RNA probably does not contain poly A. In addition, there is no significant difference in the RNA synthesis as indicated by gel electrophoresis between pineals that either have or have not been treated with isoproterenol.

In a similar manner, there appears to be no significant difference in protein synthesis between pineals which either have not been treated with isoproterenol (little NAT activity), treated with isoproterenol (NAT activity increased 30-fold), or isoproterenol treated followed by the β-receptor antagonist propranolol (reduces the induced NAT activity by 90%). This is evidenced from disc gel electrophoresis of $^{3}H/^{14}C$ labeled pineal proteins and autoradiographs of ^{35}S-methionine labeled proteins from pineals treated as above. In addition there is no significant difference in the phosphoprotein patterns of pineals exhibiting wide differences in pharmacologically altered NAT activity when the cultured pineals were incubated in the presence of ^{33}P-phosphate.

Pineal protein kinase activity was stimulated 2-4 fold within 20 minutes of isoproterenol addition to cultured glands. Both cAMP dependent and independent kinase activities increased during this time period. Protein kinase activity decreased at times later than 20 minutes after isoproterenol treatment but did not return to basal (- isoproterenol) levels through the 180 minutes of the experiment. The kinase activity was dependent on the presence of histone Type II-A and displayed little activity toward endogenous protein.

Significance to Biomedical Research and Institute Program: It may be concluded from these studies that cAMP may exert an affect on pineal NAT activity by stimulating protein kinase which in turn may influence subtle changes in transcription (RNA synthesis) and translation (protein synthesis). There seem to be no significant changes in pineal RNA, protein or phosphoprotein synthesis during the isoproterenol induction of NAT activity. The sequence of events by which neuronal impulses transmitted from the eye to the pineal may be translated into changes in enzyme activity may be hypothesized as follows:



Proposed Course of Project: The following experimental approaches will be undertaken:

1. Further definition of the role of protein kinase during the induction of NAT activity.
    a. Purification of brain protein kinase to prepare an antibody to use as a probe of pineal protein kinase activation during isoproterenol treatment.
    b. Detail the subtle changes in phosphoprotein patters resulting from the isoproterenol stimulation of pineal protein kinase.
    c. Study the physio-chemical changes in pineal protein kinase during isoproterenol treatment.

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2. Define the primary event leading to an increase in pineal NAT activity.
   a. Use purified liver NAT to prepare an antibody for use as a probe in pineal NAT synthesis.
   b. Distinguish between the primary translation of an active NAT vs the primary translation of modifying enzyme(s) which secondarily activate inactive NAT protein.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01854-05 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization of Human and Bovine Dopamine-β-Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert C. Rosenberg	Staff Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI,NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

SUMMARY OF WORK (200 words or less - underline keywords)

The long term objective of this project is to characterize and understand the mechanism of action and regulation of dopamine-β-hydroxylase (DBH). The immediate goals have been to characterize the species of DBH found in human plasma and to investigate the mechanism of action of various chemical and pharmacological inhibitors of DBH.

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PHS-6040
(12-75)

Objectives: The long term objective of this project is to characterize and understand the mechanism of action and regulation of dopamine-β-hydroxylase (DBH). The immediate goals have been to characterize the species of DBH found in human plasma and to investigate the mechanism of action of various chemical and pharmacological inhibitors of DBH.

Methods: 1. Characterization. Gradient ultracentrifugation and gel filtration have been and are being used to study the multiple forms of DBH found in human plasma.

2. Mechanism. Steady state kinetics techniques are being used to study the reaction of DBH with its substrate in the presence of chemical activators and inhibitors.

Major Findings:

Characterization: Sedimentation of whole plasma on linear sucrose gradients has shown that there are two species of DBH in plasma. The major component sediments identically to bovine DBH while the minor component has a sedimentation coefficient significantly lower than that of the major form. These forms do not appear to be in equilibrium. We find no evidence that a third species having a significantly higher sedimentation coefficient than bovine DBH exists in plasma. Other laboratories have reported such species in preparations of DBH obtained from pheochromocytoma tissue. Our results indicate that this third species is probably an artifact of the enzyme purification procedure and is thus of no physiological significance. Gel filtration studies of whole plasma also reveal two forms of DBH. Analysis of the data from both the gel filtraton and the sedimentation experiments shows that DBH is a highly assymetric molecule. Human DBH, in contrast to bovine DBH is found to exist in both dimeric and tetrameric form, both of which are enzymatically active.

Mechanism: We have been studying the kinetics of the DBH catalyzed conversion of tyramine to octopamine with $Na_4Fe(CN)_6$ serving as the electron donor. $Na_4Fe(CN)_6$ was chosen, since when it is used as the electron donor, catalase is not required to protect the DBH from inactivation as is the case with ascorbate. We have found using this system that furmarate is a true activator of DBH. We have also found that copper (II) can significantly activate DBH when $Fe(CN)_6^{4-}$ is used as the electron donor. Other divalent cations have no effect. Preliminary results indicate that this effect is not due to reincorporation of copper into inactive DBH produced during the enzyme purification procedure.

The activation of DBH by copper (II) in the presence of $Fe(CN)_6^{4-}$ is potentially useful as part of an *in vitro* assay for DBH levels in tissues. The assays currently used for this purpose are hampered by poor recoveries of enzymatic activity. In these assays, which use ascorbate as the electron donor, copper (II) is used to counteract endogenous inhibitors of DBH. However, in the presence of ascorbate, copper (II) also inhibits DBH at the concentration needed to overcome the endogeneous enzyme inhibitors. With $Fe(CN)_6^{4-}$ as the electron donor, however, much higher levels of copper (II) can be reached before any

inhibition of DBH activity is observed. Thus substitution of $Fe(CN)_6^{4-}$ for ascorbate in assay for DBH in tissue should provide a simpler and more reliable procedure.

Significance to Biomedical Research and Institute Program: DBH is essential for the function of adrenergic neurons and chromaffin cells since it catalyzes the final step in the synthesis of the neurotransmitter, norepinephrine. DBH has been found within the synaptic and chromaffin granule vesicles, and upon nerve stimulation it is released along with the neurotransmitter by an exocytotic process. Hyperactivity of the sympathetic nervous system has been implicated in the development or maintenance of essential hypertension in man and experimental forms of hypertension in animals. Detailed molecular knowledge of the structure, function and regulation of DBH is important for a complete understanding of the functioning of the adrenergic nervous system in both normal and disease states. There is a large body of work on the in vivo inhibition of DBH by various chelating and thiol compounds, some of which have been used as antihypertensive agents in man. A detailed understanding of the mechanism by which these inhibitors modulate DBH activity can provide valuable information on the functioning of the native enzyme as well as suggestions for potentially useful new antihypertensive agents.

Proposed Course of Project: Work on characterizing human DBH in plasma will continue. The elucidation of the mechanism of the activation of DBH by copper (II) observed with $Fe(CN)_6^{4-}$ as electron donor will be persued. An assay system for DBH in tissue based on this effect will also be developed. Simultaneously, the mechanism of action of various chemical and pharmacological inhibitors and activators of DBH will be studied.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01859-05 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Characterization of the Active Site(s) of Dopamine-β-hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gustavus A. Walker	NIH Postdoctorate Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI
	Hideo Kon	Research Chemist	LCP NIAMDD

COOPERATING UNITS (if any)
NIAMDD, Laboratory of Chemical Physics

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

SUMMARY OF WORK (200 words or less - underline keywords)

It is the objective of this project to determine the number of active sites on dopamine-β-hydroxylase, a copper protein from bovine adrenal medulla. This information will be obtained through a determination of the redox potential of dopamine-β-hydroxylase and by determining the number of electrons transferred as a unit through potentiometric titrations. Electron paramagnetic resonance will be used to monitor the oxidation state of the copper present in the enzyme.

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Objectives: The objective of this project is to determine the number of active sites on dopamine-β-hydroxylase (DBH) indirectly by first determining the total number of electrons transferred when DBH goes from the physiologically reduced state to the physiologically oxidized state, and secondly, by determing the number of electrons transferred as a unit through potentiometric titrations.

Methods: DBH was isolated from bovine adrenal medulla. The enzyme gave a single band with disc gel electrophoresis and was judged to be homogeneous. Dichloroindophenol, tetramethyl-p-phenylenediamine, hydroquinone and ferricyanide were selected as suitable redox mediators. Electron paramagnetic resonance was used to monitor the oxidation state of the copper present in DBH.

Major Findings: Using EPR to monitor the oxidation state of the copper present in DBH, we have been able to establish equilibria between the protein bound copper and a hydroquinone - quinone couple. These studies indicate that the midpoint potential for DBH at pH 7.0 is +307 ± 12 mv. Potassium ferricyanide or the substrate, tyramine, was used to establish the fully oxidized form of the enzyme while ascorbate was used to completely reduce the enzyme.

The copper content of several DBH preparations has been analyzed chemically and by EPR estimation and has been shown consistently to approach 4 copper atoms per tetramer of DBH. Virtually all of this copper can be reduced by ascorbate and oxidized in the presence of substrate. A titration of ascorbate reduced DBH by ferricyanide showed that at least 4 equivalences of electrons per tetramer of DBH could be removed. It seems unlikely then that there would be only one active site per tetramer of DBH.

Corroborating evidence to support two active sites per DBH tetramer was provided by a fellow worker in our laboratory who found that under certain conditions active dimers of DBH could be isolated. In addition, the subunits of DBH appear to be identical. Two dimensional peptide maps have been made of cyanogen bromide treated DBH and the results have been consistent with the number of peptides expected (∿12) if there were 10 to 11 methionine residues per (identical) subunit. The results of dansylation experiments, which indicated that a serine residue was the only N-terminal amino acid on DBH, offered further confirmation that this enzyme consists of one type of peptide.

Other interesting results which we have observed:

1. Hydroquinone will support beta hydroxylation of tyramine by DBH but only about 3% as efficiently as ascorbate under the conditions which we used.

2. The substrate, tyramine, causes superhyperfine splitting in the EPR spectrum of DBH as well as significant line narrowing. This superhyperfine splitting associated with the peak at the $g_{\perp}$ region could be due to an interaction of unpaired electrons with nitrogen surrounding the copper atoms. Either the nitrogen from the substrate amine group or nitrogen from amino

acid residues (e.g., histidine) could effect an interaction with the copper. However, data from nuclear magnetic resonance experiments show that tyramine does not affect the proton relaxation rate enhancement by protein bound copper. A more likely explanation for the substrate induced EPR spectrum changes would be a change in protein ligand conformation around the copper center(s). Quite possibly nitrogen ligands from histidine residues are brought in close juxtaposition to the copper atoms upon substrate binding.

Significance to Biomedical Research and Institute Program: Dopamine-β-hydroxylase catalyzes the final step in the synthesis of the neurotransmitter norepinephrine. Upon nerve stimulation DBH together with norepinephrine is released by an exocytotic process from sympathetic nerve endings and adrenal vesicles to appear in the serum of man. The release of DBH and catecholamines is a process intimately involved in the mechanism of nerve transmission of impulses. In order to fully understand the process of nerve impulse transmission and the physiological effects of released DBH a complete elucidation of the molecular properties of this enzyme, especially the active site(s), is necessary.

Proposed Course of Project:

1. Re-examine the reported effect of fumarate on the copper EPR spectrum of DBH and explore more fully the substrate induced superhyperfine splitting in the $g_{\perp}$ region of the spectrum.

2. Attempt a potentiometric titration of DBH to determine more precisely the midpoint potential of DBH as well as the number of electrons transferred to DBH as a unit.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01861-04 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Tryptophan Hydroxylase Depletion by Chlorinated Amphetamines and Other Drugs (revised title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Margaret Walker	NIH Postdoctorate Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI
	Hans Baumgarten	Visiting Scientist	HE NHLI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study was to examine the effect of chlorinated amphetamines and related drugs on rat brain tryptophan hydroxylase. The time course of tryptophan hydroxylase depletion by p-chloromethamphetamine (PCMA) was followed in various brain regions. It was found that in regions rich in serotonergic nerve endings there was 30% inhibition after only 3 hours, and maximum inhibition occurred at approximately 24 hours. In brain regions containing cell bodies of serotonergic neurons inhibition of tryptophan hydroxylase was not seen until 24 to 48 hours after injection. Animals pretreated with chlorimipramine did not show tryptophan hydroxylase inhibition after PCMA treatment. Also, tyrosine hydroxylase activity was unaffected. These results suggest that PCMA is taken up at serotonergic nerve endings specifically, and has a neurotoxic effect. The delayed effect on cell body areas may be due to retrograde degeneration of axons and cell bodies.

Chlorphentermine, an appetite suppressant structurally similar to PCMA, had no effect on tryptophan hydroxylase activity.

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PHS-6040
(12-75)

Objectives: Tryptophan hydroxylase is the rate-limiting enzyme in the synthesis of serotonin. Inhibition of tryptophan hydroxylase activity by chlorinated amphetamines has been shown to be one reason for the serotonin depletion caused by these drugs. Because tryptophan hydroxylase inhibition and serotonin depletion are very long lasting despite the short half-life of these drugs in the body, it was thought that the chlorinated amphetamines might have a neurotoxic effect similar to that caused by 5,6- or 5,7-dihydroxytryptamine. Since p-chloromethamphetamine (PCMA) has been used as an antidepressant in the past, and since the structurally similar drug chlorphentermine is still in use as an appetite suppressant, it seemed necessary to further examine their effects on serotonergic neurons.

Methods: Male Sprague-Dawley rats (150 to 200 gm) were given a single i.p. injection of either saline, PCMA (10 mg/kg) or chlorphentermine (10 mg/kg). At various time intervals the rat brains were removed and dissected as described by Victor, Baumgarten and Lovenberg (J. Neurochem. 22:541, 1974). Brain regions were stored in liquid N_2 until assayed, and tryptophan and tyrosine hydroxylase were assayed as described in previous reports.

For the lithium chloride studies, subcutaneous injections of saline or lithium chloride (each 5 meq/kg) were given daily. At various time intervals the brains were removed and assayed as described above.

Major Findings: Although chlorinated amphetamines have been shown to cause an immediate and long-lasting inhibition of tryptophan hydroxylase activity, a comparison of the effects in various brain regions had not been done. In our studies we observed the following:

1. Effect of PCMA

a. When the time course of tryptophan hydroxylase inhibition was examined in brain regions rich in serotonergic nerve endings (i.e. the septum, striatum and cerebral cortex) there was approximately 30% inhibition after only 3 hours, and maximum inhibition occurred at approximately 24 hours.

b. In brain regions containing cell bodies of serotonergic neurons (the mesencephalic tegmentum and pons medulla) inhibition of tryptophan hydroxylase was not seen until 24 or 48 hours after injection of PCMA.

c. Animals pretreated with chlorimipramine, a serotonergic uptake inhibitor, did not show tryptophan hydroxylase inhibition in any region at either 3 or 6 hours following PCMA treatment (longer time intervals were not examined).

d. Tyrosine hydroxylase activity was not significantly affected by PCA or PCMA.

The above findings suggest that PCMA is being taken up into serotonergic neurons specifically. It appears to be concentrated at the nerve endings, where it has an immediate effect. The delayed effect on cell body areas may be due to retrograde degeneration of axons and cell bodies.

2. Effect of Chlorphentermine.

The appetite suppressant chlorphentermine is structurally similar to PCMA, the only difference being a second methyl group on the α carbon. It is an effective inhibitor of serotonin uptake, although it does not cause a depletion of serotonin in whole rat brain. We found that chlorphentermine had no effect on tryptophan hydroxylase activity in any of the brain regions studied.

Significance to Biomedical Research and Institute Program: 1) The use of chlorinated amphetamines as antidepressants in the past and the fact that structurally related drugs are presently being used as appetite suppressants make it essential to determine the mode of action of these drugs and their possible neurotoxicity.

2) Compounds which can selectively inactivate serotonergic neurons would be useful tools in elucidating the role of serotonin in the brain. PCMA does not completely inactivate tryptophan hydroxylase. It shows a different pattern of inhibition than 5,7-dihydroxytryptamine, for instance, and therefore might be used in combination with this or other drugs to further deplete serotonin.

Proposed Course of Project:

1. Since PCMA caused an immediate (i.e., 3 hr) loss of tryptophan hydroxylase activity in the nerve ending regions, it is of interest to know whether the enzyme is inactivated or whether actual destruction of some of the serotonergic nerve endings occurs at this time. We are currently purifying tryptophan hydroxylase from rat tegmenta in order to prepare antibodies against it. Using the radioimmune assay we will attempt to determine whether tryptophan hydroxylase is present but inactivated or whether enzyme levels are actually decreased.

2. It has recently been reported that lithium chloride causes a biphasic alteration in rat brain serotonin synthesis by altering both tryptophan uptake and soluble tryptophan hydroxylase levels (Knapp and Mandell, J. Pharm. Exp. Ther. 193: 812-823, 1975). Tryptophan hydroxylase levels had decreased in both the midbrain (which contains cell bodies of serotonergic neurons) and the striatum (which contains serotonergic nerve endings) by 24 hours. In view of our previous work on tryptophan hydroxylase depletion, it is of interest to examine the effect of lithium chloride more extensively and to determine the reason for this loss in tryptophan hydroxylase activity (i.e. - decreased enzyme synthesis, inactivation, increased degradation). A study is now underway to see if our laboratory can reproduce the results seen by Knapp and Mandell. Since the lithium chloride levels used in the above study have been reported to be toxic by some workers, it is important to determine whether there is a long-term effect on tryptophan hydroxylase after treatment is discontinued.

Publications:

1. Lovenberg, W., Walker, M.N. and Baumgarten, H.G.: Chlorinated amphetamines: Drugs or toxins? Agents and Actions 5: 501, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01862-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Purification of an Enzyme which N-acetylates Serotonin from Rat Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Morrissey	Staff Fellow	HE NHLI
OTHER: W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

An enzyme which is capable of N-acetylating serotonin or tryptamine was routinely purified about 350-fold from rat liver high speed supernatants. The apparent K_m for the substrates acetyl CoA and tryptamine were found to be similar to that of an enzyme performing a similar function in the rat pineal gland. The apparent molecular weights of the liver and pineal enzymes were also found to be essentially the same. The pH optimum for the liver enzyme was in the vicinity of pH 8.5 but the optimum of the pineal enzyme was pH 6.5.

817

PHS-6040
(12-75)

Objectives: The purpose of this investigation is to purify and characterize an enzyme from rat liver which is capable of N-acetylating serotonin. The purified enzyme would be used to generate an antibody in rabbits. The antibody would in turn be used as a probe for studying the biosynthesis of serotonin-N-acetyltransferase in the liver and pineal gland of rats.

Methods: N-acetyltransferase activity was determined by a sensitive radiochemical technique using tryptamine as an acetate acceptor. Conventional protein purification techniques of ammonium sulfate fractionation, DEAE cellulose, and Sephadex gel filtration were used throughout.

Major Findings: The liver enzyme was purified 350-fold from highspeed supernatant fractions by ammonium sulfate fractionation, DEAE cellulose chromatography, and Sephadex G100 gel filtration. The enzyme is not completely pure at this stage, however. The enzyme is quite labile and is somewhat stabilized by the presence of sulfhydryl reagents. The K_m for substrates and molecular weights of the liver and pineal enzymes were determined on crude high speed supernatants due to the extreme lability of the pineal enzyme. The K_m for the substrate Acetyl Co A and tryptamine were similar (approximately 0.06 mM and 0.5 mM, respectively) and the apparent molecular weight by gel filtration was also similar (25,000) for the enzyme from both the liver and pineal. This suggests some similarity between the two enzymes, however, the pH optimum of activity are quite different. The liver enzyme is most active at pH 8.5 while the pineal enzyme is active at pH 6.5.

Significance to Biomedical Research and Institute Program: The isoniazid-N-acetyltransferase enzyme of the liver may be important in drug metabolism while the pineal enzyme is the highly regulated rate limiting step in melatonin biosynthesis. An antibody to the liver enzyme would be an ideal tool in the study of N-acetyltransferase regulation in the liver and pineal gland.

Proposed Course of Project: Completely purify the N-acetyltransferase enzyme from rat liver.

1. Study the kinetic parameters of the enzyme
2. Study the physical parameters of the enzyme
3. Prepare an antibody against the enzyme

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01863-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effects of Morphine on Blood Platelet Adenyl Cyclase Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Morrissey	Staff Fellow	HE NHLI
OTHER:	W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The blood platelet has been considered as an accessible model serotonergic neuron. Additionally, it is thought that morphine inhibits the adenylate cyclase activity of neural cells causing the affected cells to produce more adenyl cyclase enzyme in order to maintain the normal activity. This investigation is concerned with characterizing the affects of morphine on the prostaglandin stimulated adenyl cyclase of blood platelets.

PHS-6040
(12-75)

Objectives: The purpose of this investigation is to purify and characterize an enzyme from rat liver which is capable of N-acetylating serotonin. The purified enzyme would be used to generate an antibody in rabbits. The antibody would in turn be used as a probe for studying the biosynthesis of serotonin-N-acetyltransferase in the liver and pineal gland of rats.

Methods: N-acetyltransferase activity was determined by a sensitive radiochemical technique using tryptamine as an acetate acceptor. Conventional protein purification techniques of ammonium sulfate fractionation, DEAE cellulose, and Sephadex gel filtration were used throughout.

Major Findings: The liver enzyme was purified 350-fold from highspeed supernatant fractions by ammonium sulfate fractionation, DEAE cellulose chromatography, and Sephadex G100 gel filtration. The enzyme is not completely pure at this stage, however. The enzyme is quite labile and is somewhat stabilized by the presence of sulfhydryl reagents. The K_m for substrates and molecular weights of the liver and pineal enzymes were determined on crude high speed supernatants due to the extreme lability of the pineal enzyme. The K_m for the substrate Acetyl Co A and tryptamine were similar (approximately 0.06 mM and 0.5 mM, respectively) and the apparent molecular weight by gel filtration was also similar (25,000) for the enzyme from both the liver and pineal. This suggests some similarity between the two enzymes, however, the pH optimum of activity are quite different. The liver enzyme is most active at pH 8.5 while the pineal enzyme is active at pH 6.5.

Significance to Biomedical Research and Institute Program: The isoniazid-N-acetyltransferase enzyme of the liver may be important in drug metabolism while the pineal enzyme is the highly regulated rate limiting step in melatonin biosynthesis. An antibody to the liver enzyme would be an ideal tool in the study of N-acetyltransferase regulation in the liver and pineal gland.

Proposed Course of Project: Completely purify the N-acetyltransferase enzyme from rat liver.

1. Study the kinetic parameters of the enzyme
2. Study the physical parameters of the enzyme
3. Prepare an antibody against the enzyme

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01863-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effects of Morphine on Blood Platelet Adenyl Cyclase Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Morrissey	Staff Fellow	HE NHLI
OTHER:	W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The blood platelet has been considered as an accessible model serotonergic neuron. Additionally, it is thought that morphine inhibits the adenylate cyclase activity of neural cells causing the affected cells to produce more adenyl cyclase enzyme in order to maintain the normal activity. This investigation is concerned with characterizing the affects of morphine on the prostaglandin stimulated adenyl cyclase of blood platelets.

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PHS-6040
(12-75)

Objectives: This investigation is designed to test the effects of morphine on blood platelet adenyl cyclase activity. Additionally, the adenyl cyclase activity of normal patients and morphine addicts will be compared to assess the long term influence of morphine on platelet adenyl clclase. Drug withdrawal will be mimicked by the inclusion of naloxone, a competitive inhibitor of morphine.

Methods: Platelet rich plasma was prepared from citrated blood by differential centrifugation. Staining of platelet rich plasma smears indicates that there is little contamination with red cells or white cells. Platelets were pre-incubated with ^{14}C-adenine in order to equilibrate the adenine nucleotide pools of the platelets prior to the addition of drugs. The cAMP resulting from drug (prostaglandin E_1) treatment of the ^{14}C-adenine labeled platelets was isolated from acidified ($HClO_4$) samples on alumina followed by Dowex 50 columns. A trace of ^{3}H-cAMP was used to monitor recovery. The adenyl cyclase activity was expressed as the percentage of total ^{14}C-adenine as cAMP.

Major Findings: The project is in its infancy and as such there are no real major findings.

Significance to Biomedical Research and Institute Program: The blood platelet may be a readily accessible and useful model of serotonergic neurons. If this is so the morphine addiction-withdrawal studies may serve as an useful framework for the investigation of other drugs on the adenyl cyclase activity of neurons.

Proposed Course of Project:

1. Assess the affect of morphine and naloxone on blood platelet adenyl cyclase activity of normal individuals.

2. Compare the levels of blood platelet adenyl cyclase activities of morphine addicts.

3. Assess the affect of naloxone treatment on the blood platelet adenyl

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01864-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Effect of Phenelzine on Tryptophan and Tyrosine Hydroxylase Activities

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Margaret Walker	NIH Postdoctorate Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI
	I. C. Campbell	Staff Fellow	LCS NIMH
	R. Colburn	Staff Fellow	LCS NIMH
	D. Murphy	Chief, Sect. Clin. Neuropharmacol.	LCS NIMH

COOPERATING UNITS (if any)
Laboratory of Clinical Science, NIMH

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Following treatment of rats with phenelzine (a monoamine oxidase inhibitor) endogenous levels of norepinephrine and 5-hydroxytryptamine in the brain show an initial increase, however there is an adaptive response and amine values return towards control values over 21 days treatment (I.C. Campbell, unpublished results). This study was undertaken to determine whether there was a compensatory decrease in tryptophan hydroxylase or tyrosine hydroxylase which would explain the corresponding drop in amine levels.

Preliminary results showed that tyrosine hydroxylase activity in the various brain regions studied was unchanged by phenelzine treatment. Tryptophan hydroxylase activity showed a slight but significant increase in several brain regions. In no region studied was there a decrease in tryptophan hydroxylase activity. We are currently investigating this phenomena further by examining 1) the kinetics of the reaction (i.e., the effect of phenelzine treatment on the K_m of the enzyme for either tryptophan or cofactor), 2) possible activation of the enzyme, 3) corticosterone levels following phenelzine treatment, to see if tryptophan hydroxylase synthesis could be indirectly induced, and 4) tryptophan levels in the brain following phenelzine treatment.

821

Objectives: Treatment of affective disorders with antidepressant drugs usually requires about two weeks to elicit a therapeutic response. Therefore, it is necessary to study the effects of chronic treatment with these drugs to explain their mode of action.

Treatment of rats with phenelzine (an antidepressant which is a monoamine oxidase inhibitor) caused an initial increase in endogenous levels of norepinephrine and 5-hydroxytryptamine. Over 21 days treatment, however, there is an adaptive response and amine levels return towards control values (I.C. Campbell, unpublished results). The purpose of this study was to examine tryptophan and tyrosine hydroxylase in various brain regions over the 21 day treatment with phenelzine, to determine if there was a compensatory decrease in enzyme activity which would account for the corresponding drop in amine levels with time.

Methods: Male Sprague-Dawley rats (∿150 gm) were given i.p. injections of saline or phenelzine (15 mg/kg/48 hr). At 1, 7, 13 and 21 days, and 6 hours after the last injection, the animals were sacrificed. Rat brains were removed and dissected as described by Victor, Baumgarten and Lovenberg (J. Neurochem. 22: 541, 1974). Brain regions were stored in liquid N_2 until assayed and all samples of a given region were assayed at the same time. Tryptophan and tyrosine hydroxylase were assayed as described in annual report Z01 HL 01865-01 HE.

Corticosterone levels were assayed in heparinized plasma as described by Gal and Millard (Biochem. Pharmacol. 17: 2193, 1968). Brain tryptophan levels were assayed by the method of Denckla and Dewey (J. Lab. Clin. Med. 69: 160,1967).

Major Findings:

1. In two separate studies there were slight but significant ($p < 0.05$) increases in tryptophan hydroxylase activity in phenelzine-treated rats over control rats. This increase was not seen in each region at each time point, and therefore the effect must be more thoroughly examined. The effect was generally seen in the mesencephalic tegmentum, the region containing the raphe nuclei. There were no points where tryptophan hydroxylase was significantly lower in phenelzine treated rats, although this was the effect which might have been expected from the decrease in 5-hydroxytryptamine levels following chronic phenelzine treatment.

2. Tyrosine hydroxylase was not affected by chronic phenelzine treatment.

3. Plasma corticosterone levels were unaffected by chronic phenelzine treatment. Therefore, the increase in tryptophan hydroxylase activity cannot be explained by an indirect of phenelzine via induction by corticosterone.

4. Some kinetics experiments have been done on tegmental tryptophan hydroxylase from rats treated 18 hrs and 5 days with phenelzine. Preliminary results suggest that there are changes in the kinetic parameters of the enzyme, however, these studies must be redone before conclusion can be drawn.

Significance to Biomedical Research and Institute Program:

1. Since antidepressant drugs may require two weeks to exert their effect, it is essential to understand the results of chronic treatment on the biochemistry of amine synthesis and catabolism. Unfortunately many of the animal studies on antidepressant drugs have examined the acute effects only.

2. Since tryptophan hydroxylase in the rate-limiting enzyme in serotonin biosynthesis, it is likely that there would be factors regulating either the amount or activity of the enzyme, and thus regulating serotonin levels. So far, it has been difficult to show any clear evidence for such mechanisms and tryptophan hydroxylase regulation remains somewhat of a mystery. It has been postulated that there is a family of regulatory mechanisms functioning to keep serotonin levels constant in the brain. This project may give more insight into these mechanisms.

Proposed Course of Project: The short-and long-term effects of phenelzine on tryptophan hydroxylase will be reexamined. In particular, kinetic studies will be repeated on both crude supernatant and partially purified enzyme. Brain tryptophan concentrations will be measured to see if they are affected by phenelzine treatment.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01865-01 HE

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Tryptophan Hydroxylase Purification and Characterization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Margaret Walker	NIH Postdoctorate Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to purify, characterize, and compare tryptophan hydroxylase from several different sources including rat mesencephalic tegmentum and mouse mast cell tumors. One aim of the project is to supply purified tegmental tryptophan hydroxylase for the production of antibodies to be used in studies on tryptophan hydroxylase regulation. A second aim is to determine the biochemical characteristics of tryptophan hydroxylase from such divergent sources as mesencephalic tegmentum, pineal gland, mast cells, and septum, and to determine the degree of identity between these.

824

Objectives: Tryptophan hydroxylase is the rate-limiting enzyme in serotonin biosynthesis. It catalyzes the reaction:

L-tryptophan + tetrahydropterin + O_2 → 5-OH-L-tryptophan + dihydropterin + H_2O

In the central nervous system of mammals tryptophan hydroxylase is synthesized in serotonergic cell bodies found primarily in the raphe nuclei of the midbrain, and it is transported to nerve endings in various regions of the brain and spinal cord. Some studies on the response of tryptophan hydroxylase levels to drugs or brain lesions have suggested that the tryptophan hydroxylase found in the septum may be synthesized there, and not in the tegmentum. Additionally, tryptophan hydroxylase is found in high concentrations in the pineal gland and in certain mast cell tumors. It is the objective of this study to purify and characterize tryptophan hydroxylase from these tissues to determine the degree of identity between them. Antibodies will also be prepared for studies on the regulation of synthesis of the enzyme.

Methods: Tryptophan hydroxylase is currently being purified from both rat mesencephalic tegmentum and mouse mast cell tumors. The procedures found to be effective include polyethylene glycol fractionation, sepharose 4B filtration and affinity chromatography using 4-phenylbutylamine (PBA) substituted sepharose. Other purification procedures attempted included DEAE-chromatography, calcium phosphate gel absorption, hydroxyapetite absorption and ammonium sulfate fractionation.

Tryptophan hydroxylase was assayed by a modification of the procedure of Friedman et al. (J. Biol. Chem. 247: 4165, 1972). Frozen tissues were weighed and homogenized with 0.05 M Tris-HCl and 0.002 M dithiothreitol (pH 7.4) in a 1:5 weight:volume ratio and centrifuged at 40,000 g for 20 minutes. The supernatant fraction was assayed for tryptophan hydroxylase activity by the method of Friedman et al. (1972) as described in Baumgarten et al. (1973) with volumes altered to give a more sensitive microassay. A 20 µl portion of the supernatant fraction was incubated with 12 µmoles Tris-HCl (pH 7.4), 0.4 µmole dithiothreitol, and 17 nmoles 6-methyltetrahydropterin in a total volume of 40 µl at 37° for 10 min. The reaction was started by the addition 10 µl of 0.002 M tryptophan. After 30 min 10 µl of 6 N perchoric acid was added to stop the reaction, and the samples were centrifuged. A 40 µl portion of the supernatant was added to 100 µl of 3 N HCl and the fluorescence of the solution was measured at 540 nm, using an excitation wavelength at 290 nm. 5-HTP standards were run at the same time. It was necessary to modify the assay further for accurate measurement of more purified tryptophan hydroxylase.

Major Findings: A major problem in the purification of tryptophan hydroxylase has been the low yields of the enzyme due to its extreme lability. During the purification from rat mesencephalic tegmentum, polyethylene glycol (PEG) fractionation (4-10% weight:volume) gave a 3 to 5-fold increase in specific activity but only 25-50% recovery of activity. Sepharose 4B filtration resulted in ∿ 15-fold increase in specific activity over the crude supernatant, but the amount of activity found in the peak fractions was only ∿ 10% of the original units.

Studies on the lability of the enzyme demonstrated a strong protective effect of catalase, both during sepharose filtration and while samples were stored at cold room temperatures. This protective effect could not be observed with bovine serum albumin.

Partially purified tryptophan hydroxylase was found to be extremely labile under standard assay conditions. The assay was modified by 1) elimination of the preincubation step, 2) cutting down the time of the reaction to 5-10 minutes and 3) adding catalase ($\sim$ 1 mg/ml) to the protein sample to be assayed. Under these conditions the specific activity of tryptophan hydroxylase subjected to PEG fractionation and sepharose 4B filtration was several-fold higher than previously estimated. Using the new assay it is possible to re-evaluate the efficiency of several purification procedures which previously appeared ineffective because the tryptophan hydroxylase activity was not being assayed optimally.

Significance to Biomedical Research and Institute Program: One of the main reasons for pursuing this project is the need for antibodies against purified tryptophan hydroxylase, so that studies on regulation can be carried out. An examination of the literature suggests that insight into much of the confusing or contradictory data on changes in tryptophan hydroxylase levels could be obtained using antibody prepared to purified tryptophan hydroxylase. In addition, characterization of tryptophan hydroxylase preparations from various brain regions and the pineal may explain why tryptophan hydroxylase activities in these regions do not respond identically to various drug treatments.

Proposed Course of Project:

Work is continuing on the purification of tryptophan hydroxylase from the sources mentioned. At the same time, kinetic studies are being carried out on partially purified enzyme to determine wwhether factors reported to stimulate crude supernatant tryptophan hydroxylase activity exert their effect on the enzyme itself or whether they have an indirect effect (i.e., while calcium has been shown to lower the K_m of the crude enzyme for its substrate tryptophan, it appears to have no effect on partially purified enzyme which already has a lower K_m for tryptophan).

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01866-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Purification of Rat Striatal Tyrosine Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Matthew M. Ames	Research Associate	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term goal of this work is the purification of rat striatal tyrosine hydroxylase. We have achieved a significant increase in specific activity via Sepharose 4B chromatography, and are currently studying hydroxylapatite chromatography. Future studies will utilize the above proceducres as well as affinity chromatography and disc gel electrophoresis. We hope to obtain purified preparations for further kinetic characterization.

827

PHS-6040
(12-75)

Objectives: Tyrosine hydroxylase has been partially purified from bovine adrenal tissue by traditional column chromatography techniques. Other methods have included preliminary treatment with trypsin followed by column chromatography methods. Human pheochromocytoma tyrosine hydroxylase has reportedly been purified via salt precipitation, disc gel electrophoresis, and sucrose density gradient treatment. However, attempts at purification of rat striatal enzyme have been much less successful--there are no literature reports of a highly purified preparation. Two of the major problems encountered in purification of striatal tyrosine hydroxylase are the instability of the enzyme and its tendency to aggregate during purification. The latter problem is illustrated by the fact that molecular weight estimates have varied from 35,000-200,000 daltons. The objectives of this study are to improve the purification procedures for isolation of rat striatal tyrosine hydroxylase, and to obtain purified preparations with enough residual activity for kinetic studies.

Methods: Preliminary steps in our purification studies have included ammonium sulfate fractionation, equilibrium dialysis, and Sephadex G-25 chromatography. Major purification steps have included Sepharose 4B column chromatography in 20 mN potassium phosphate buffer (pH 6.0), hydroxylapatite chromatography with stepwise elution in potassium phosphate buffer (varying concentration gradients), and hydroxylapatite chromatography with continuous elution (gradients from 20 mM to 300-1000 mM). Enzymatic activity has been determined by the standard tritium release assay under optimal conditions, and proteins determined by the Lowry method. Visual indication of purity has been studied using disc gel electrophoresis under a variety of conditions.

Major Findings: We have been involved in improvements of existing tyrosine hydroxylase purification techniques such as ammonium sulfate fractionation (reduction of percentage from 80 to 60) and the addition of Triton X-100 in the homogenization buffer. These minor modifications have increased the yield in the early purification steps. We have achieved a 20 to 5-fold increase in specific activity after Sepharose 4B column chromatography. After many difficulties with hydroxylapaptie chromatography, we have just recently been able to recover significant enzyme activity with some purification. This was made possible by the use of steeper concentration gradients and larger column dimensions. Disc gel electrophoresis has suggested that other reports of an essentially pure enzyme after hydroxylapatite chromatography may be too optimistic. We have also synthesized an affinity column with a Sepharose 4B resin, 6 carbon spacer arm, and tyrosine, hopefully linked through the aromatic portion of the molecule rather than through the amino function. Preliminary kinetic studies suggests that with mercaptoethanol as the reducing system, the partially purified (Sepharose 4B) enzyme may not be subject to further activation by phosphorylation, as reported in the literature.

Significance to Biomedical Research and Institute Program: Characterization of the enzyme which is rate-determining in the production of catecholamines in the central nervous sytem would enhance the understanding of the role and regulation of these enzymes in the central nervous system. Purification of tyrosine hydroxylase is a necessary prerequisite to such a characterization and would therefore be a significant contribution to biomedical research.

Proposed Course of Project: We plan to continue our present studies with Sepharose 4B and hydroxylapatite chromatography, as well as studying the application of an affinity column to our purification techniques. We also hope to better employ disc gel electrophoresis in interpretation of the purification studies. While we have no specific plans to examine other techniques, we anticipate expanding our methodology in further purification studies.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01867-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Short Term Regulation of Tyrosine Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert C. Rosenberg	Staff Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmcol.	HE NHLI
	Eleanor Bruckwick	Research Assistant	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying the kinetic properties of tyrosine hydroxylase from rat striata in order to elucidate the mechanism of its short term regulation.

830

PHS-6040
(12-75)

Objectives: The objective of this project is to study the mechanisms involved in the short term regulation of tryrosine hydroxylase.

Methods: Steady state kinetics techniques are being used to study the properties of tyrosine hydroxylase in crude homogenates of the striatal area of rat brains. The standard tritium release assay for this enzyme is being employed in all experiments.

Major Findings: The apparent K_m of tyrosine hydroxylase (TH) for its cofactor has been shown in both this and other laboratories to be sensitive to various environmental factors such as protein phosphorylation or added anions. The kinetics of tyrosine hydroxylase system are complicated, since three substrates are involved. The usual approach in studying such a system is to use saturating concentration of all but one of the substrates. Unfortunately with TH this approach cannot be used since substrate inhibition by tyrosine is observed. To circumvent this problem we are studying this system kinetically at subsaturating levels of the various substrates.

There has been some question in the literature over whether TH follows a sequential or ping-pong mechanism with respect to the pterin cofactor and tyrosine. Our results are consistent only with a sequential mechanism. We find that under conditions which promote protein phosphorylation the K_m of TH for its natural cofactor is reduced by a factor of 4 (0.3 mM to 0.07 mM). These same incubation conditions has only a small effect on the K_m for tyrosine. Preliminary experiments with oxygen as the varied substrate indicate that atmospheric concentrations of oxygen may not be saturating ($K_m \sim$ 10-15%). If confirmed these findings mean that oxygen levels in tissue may also play a significant role in regulating TH activity *in vivo*.

Significance to Biomedical Research and Institute Program: Tyrosine hydroxylase is the rate limiting enzyme for the synthesis of catecholamines in the central nervous system. Factors which effect the activity of TH can influence the levels of catecholamines. Regulation of TH activity by product or feedback inhibition is well known. It now appears that there are other regulatory modes for this enzyme as well. Studies of these effects can help us elucidate and understand the multivaried response of catecholamine containing neurons to external stress.

Proposed Course of Project: We shall continue our kinetic studies on the activation of TH using unpurified rat striatal tissue homogenate as the source of the enzyme. In particular we shall study in detail the kinetics of TH with oxygen as one of the varied substrates in order to determine whether hypoxia can signifcantly effect TH activity. We shall also complete our kinetic studies on the effects of anions such as acetate on the affinity of the enzyme for its other substrates.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01868-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulation of Tyrosine Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pauline Lerner	Postdoctorate Fellow	HE NHLI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Tyrosine hydroxylase is an important regulatory enzyme for the biosynthesis of catecholamines. When a single dose of haloperidol, a dopamine antagonist is administered to rats, there is a rapid increase in the activity of tyrosine hydroxylase in the striatum. The increase in activity is mediated by an increase in the affinity of the enzyme for its pterin cofactor. We have found that when a striatal extract from haloperidol treated rats is mixed with a striatal extract from control rats, the tyrosine hydroxylase activity is much more than additive. This suggests that there may be an activator of tyrosine hydroxylase present in extracts from treated rats. We have also studied the effect of chronic haloperidol treatment on rat striatal tyrosine hydroxylase. After seven or more daily injections of haloperidol, an additional dose of the drug causes an immediate increase, followed by a sharp decrease, to a level below that of controls, in striatal tyrosine hydroxylase activity. This may represent a compensatory mechanism by which the enzyme adjusts to tissue levels of dopamine.

PHS-6040
(12-75)

Objectives: Tyrosine hydroxylase is the rate limiting enzyme in the biosynthesis of the catecholamine neurotransmitters. It is known that blockade of central dopaminergic receptors with neuroleptics, such as phenothiazines and butyrophenones, leads to an increase in dopamine turnover. Dopamine synthesis is increased via a rise in the activity of tyrosine hydroxylase. The stimulation of the enzyme is mediated by an increase in the affinity of the tyrosine hydroxylase for its pterin cofactor. We are interested in studying the regulation of tyrosine hydroxylase in rats receiving acute or chronic injections of haloperidol, a butyrophenone type drug. This is of particular interest in light of recent findings which suggest that there may be significant differences between the long term and short term effects of neuroleptic treatment on central dopamine metabolism in humans.

Methods: Haloperidol or saline is administered to rats by intraperitoneal injection. After appropriate intervals of time, the rats are sacrificed and the striata are removed rapidly. Tyrosine hydroxylase is assayed in striatal extracts by a tritium release method.

Major Findings: Our short term experiments with haloperidol confirm and extend the findings of others. One hour after injection of haloperidol, there is an increase in striatal tyrosine hydroxylase activity. This stimulation is effected by an increase in the affinity of the enzyme for its pterin cofactor. In addition, we have found that mixing of striatal extracts from treated and untreated rats gives an enzyme activity which is much greater than additive. This suggests that there may be a transferable activating factor for tyrosine hydroxylase in extracts from drug treated rats.

When haloperidol is administered chronically, and tyrosine hydroxylase is measured one hour after the last injection, the usual increase in enzyme activity is seen. However, when the drug is administered chronically, and the enzyme is assayed 23 hours after the last injection, the enzyme activity is decreased significantly below control levels. In this case, there is an apparent decrease in the affinity of the enzyme for its pterin cofactor. These changes may indicate a compensatory response of tyrosine hydroxylase to tissue levels of dopamine.

Significance to Biomedical Research and Institute Program: Although neuroleptic drugs have constituted a major mode of therapy for schizophrenia in the last twenty years, the precise biochemical mechanism by which these drugs exert their antipsychotic effects is not known. A major site of action of these drugs appears to be central dopaminergic nerve terminals. The drugs are known to affect the turnover of dopamine and the activity of the dopamine biosynthetic enzyme, tyrosine hydroxylase. This project is directed towards obtaining a better understanding of the regulation of this enzyme by neuroleptic drugs.

Proposed Course of Project: We are interested in obtaining more information on the putative activator of tyrosine hydroxylase seen in striatal extracts from drug treated rats. We also plan to extend our studies on chronic haloperidol treatment to non-striatal dopaminergic sites in the brain.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01869-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Problems Associated with the Study of Uptake in Rat Heart

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald F. Bogdanski Pharmacologist HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

A difficult problem for the studies of amine uptake by nerve endings in peripheral organs is the small proportion of tissue that actually participates in the uptake phenomenon. For example, slices of rat ventricle contain less than 0.1% of adrenergic nerve endings. Hence, even poor uptake by the large bulk of muscle tissue affects the results of uptake studies. Conversely, if the concentration of amine in heart reached equality with that in the extracellular fluid, then the uptake into nerve tissue would be at least 1000:1 to raise the overall tissue concentration to 2:1. To increase the precision of uptake studies using heart slices, some estimate of the uptake by non-carrier mediated uptake in the tissue is required to estimate uptake by carrier-mediated mechanisms in neuronal tissue. We have been using a tissue: medium ratio of 0.8 as an adjustment factor for this purpose. More data is now available and the problem was re-investigated for current studies.

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Objectives: To estimate the amount of extraneuronal uptake of ^{3}H-norepinephrine in rat heart slices.

Major Findings:

The estimation of extracellular volume

Rat heart ventricle slices were pre-incubated in Krebs-bicarbonate solution at 37°. This procedure tends to re-establish homeostasis after the injurious procedure of slicing. Carbon^{14}-labelled inulin was then introduced into the medium, and the slices were incubated for various lengths of time. Accumulated radioactivity was estimated by procedures commonly used in this laboratory. Some of the slices were transferred to fresh media for the estimation of retention of radioactivity.

The slices accumulated C^{14}-inulin to a constant tissue: medium ratio of 0.54 between 120 to 150 min of incubation. Curves of uptake vs time of incubation suggested that more than one process was active during this time. The efflux of accumulated radioactivity was studied after eight minutes of uptake, when it appeared as though most of the radioactivity had been accumulated by a single process. The half-time of retention was 3.6 minutes, with <10% remaining. If there were only one (extracellular) compartment, then uptake should occur with a similar half-time, and uptake should have been 94% complete after 4 half-times (14 min). However, uptake was only 62% complete after 14 min. It is concluded that inulin enters more than one compartment. After 15 min, the inulin space is about 37% of the tissue, but this space varies with the thickness and structural integrity of the slices. Nearly all of the inulin space (33% of tissue) is extracellular at this time.

Further analysis was not attempted at present, but it can be assumed that the inulin space includes intracellular space in muscle fibers that are likely to be transected in at least two places, and perhaps damaged otherwise.

The actual non-neuronal space in 0.5 g of heart slice which is accessible to ^{3}H-norepinephrine (^{3}H-NE) has been represented as 0.8 times 0.5 ml (∿g) of extracellular fluid. A list of accumulation ratios indicate that this approximation is not greatly in error. These ratios are listed as follows:

Incubation Medium	Accumulation Ratio 0.5 g tissue/0.5 g medium 15 min incubation
Na^{+}-free, choline-krebs-bicarbonate	1.3
Krebs-bicarbonate, 17 µM cocaine	1.2
Na^{+}-free, lithium-krebs-bicarbonate	1.0
Na^{+}-free, choline-Krebs-bicarbonate 100 µM cocaine, 0°	0.9
Krebs-bicarbonate, 1450 µg/ml ℓ-NE	0.83

The first three media could permit some carrier mediated uptake, whereas the latter two should not (see below). Our original use of 0.8 is, therefore, not grossly in error. Moreover, in the absence of carrier mediated uptake, ^{3}H-NE can reach compartments that are inaccessible to C^{14}-inulin.

It was desired to calculate A_n, the accumulation of ^{3}H-NE exclusive of extracellular fluid and passive distribution in muscle and nerve. The following procedures were employed: Eliminate carrier mediated transport into nerve tissue. Preincubate slices 40 min in Krebs-bicarbonate medium, add cocaine to a final concentration of 100 µM and incubate 20 min more. Transfer the slices to ice cold, Na^{+}-free Krebs-bicarbonate medium containing 100 µM cocaine and ^{3}H-ℓ-NE at 1.4 nM .

2. Preincubate slices 50 min in Krebs-bicarbonate medium. Add ℓ-NE at 750 µM (about 3,500 x the K_m).

In these two experiments, carrier mediated uptake is prevented (experiment 1) or rendered nearly insignificant relative to the uptake in the presence of a huge concentration of ℓ-NE (Experiment 2).

An analysis of accumulation in rat heart ventricle slices follows: Ordinary uptake occurs in nerve (N) + Muscle (M) + extracellular space (XS). By subtraction: $(A_n + A_M + A_{xs}) - (A_M + A_{xs}) = A_n$ (Eq. 1) where A_N is the carrier mediated accumulation of ℓ-NE in nerve tissue, the amount of which we do not know. Therefore, A_N is expressed in forms of cpm/0.5 g heart tissue.

The quantity $(A_M + A_{xs})$ is expressed in terms of the quantity of radioactivity in the incubation medium. This quantity was calculated in experiments in which carrier mediated uptake in nerve tissue was eliminated by either procedure 1 or 2. Thus,

$$\frac{\text{cpm x 0.5 g}}{\text{tissue wt.(g)}} = (A_M + A_{xs}) \quad \text{Eq. 2}$$

In experiment 2 above,

$A_N = 0$, by definition, and $(A_N + A_M + A_{xs})$ as well as $(A_M + A_{XF})$ = 0.83 x cpm Fluid 0.5 ml n 0.5 g.

In an ordinary experiment in which carrier mediated uptake can occur, the value for N can be estimated by substituting the total uptake and the term 0.83 cpm fluid (0.5 ml) in equation 1 as follows:

$$A_N = \frac{\text{cpm x 0.5 g}}{\text{t.wt.(g)}} - (0.83 \text{ cpm Fluid } 0.5 \text{ ml}) \quad \text{Eq. 4}$$

In using this formula, the accumulation (A_N) of an amount of radioactivity equal to that contained in 0.5 ml medium actually represents an accumulation of radioactivity at least 1000 times that in the medium.

The quantity of 0.5 g tissue represents 33% extracellular medium. To estimate tissue to medium ratios, the quantity A_N can be multiplied by

$$\frac{0.5}{0.67 \text{ x } 0.5} = 1.5$$

Significance to Biomedical Research and to the Institute Program: This report helps to resolve technical problems in studies of transport in nerve endings. These studies are problematical because of the innate quantity of actively transporting tissue in a large mass of non-transportic tissue. These findings help us to understand associated phenomena occurring during transport which can affect the results of experiments.

Proposed Course of Project: The project is terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01870-01 HE

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Vascular Protein Synthesis in the Spontaneously Hypertensive Rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLI
OTHER:	Teruhiro Nakada	Visiting Scientist	HE NHLI
	Yukio Yamori	Visiting Scientist	HE NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

SUMMARY OF WORK (200 words or less - underline keywords)

A system to measure the incorporation of lysine in vivo into collagen, elastin, and non-collagen proteins has been established. Spontaneously hypertensive rats have much greater rates of lysine incorporation into non-collagen protein of the mesenteric arteries than do normotensive control rats. This apparent increased synthesis of non-collagen protein appear to be controlled by the sympathetic nervous system, since denervating the vessels or treating the animals with a ganglionic blocker (hexamethonium) reduced in incorporation rates to the control level. Conversely, hydralazine which is a very effective hypotensive agent but actually causes an increase in sympathetic outflow did not reduce the rate of incorporation. Since these observations were made in very young rats it is suggested that increased protein syntheses in the small vessles may be one of the pathogenic factors in spontaneous hypertension.

PHS-6040
(12-75)

Objectives: The purpose of this work was to determine whether sympathetic nerves have a trophic effect on the smooth muscle of the small arteries. The spontaneously hypertensive rats (SHR) which exhibit substantially higher rates of non-collagen protein synthesis in the mesenteric arteries than do their normotensive controls (WKR) were used as the model system. It was, therefore, of interest to determine the effect of sympatholytic agents on blood pressure and amino acid incorporation into non-collagen protein.

Methods: Six week old SHR and age matched WKR were divided into 5 groups of 10 and treated for 2 weeks. Group I, control SHR, Group II SHR received hydralazine (80 mg/liter in the drinking water), Group III SHR treated with 50 mg/kg hexamethonium twice daily for two weeks, Group SHR splanchnicotomized at 6 weeks of age and Group Control WKR. At 8 weeks of age the animals each received a pulse of tritiated lysine 2 hours before sacrifice and the elastin collagen and non-collagen protein extracted from the heart, aorta and mesenteric arteries as described in previous reports.

Major Findings: Hydralazine or hexamethonium or splanchnicotomy effectively arrested the development of hypertension for two weeks in young spontaneously hypertensive rats (SHR). The rate of incorporation of ^{3}H-lysine into non-collagenous proteins *in vivo* of the heart, aorta and mesenteric arteries was determined in the treated SHR, as well as control SHR and normal Wistar/Kyoto (WK) rats. The lysine incorporation into the non-collagenous protein of mesenteric arteries was increased 2-fold in 8-week-old SHR as compared with WK rats. The elevated lysine incorporation in the SHR was abolished by treatment with hexamethonium or by splanchnicotomy, but was not affected by treatment with hydralazine. No significant changes in collagen and elastin synthesis occurred following antihypertensive therapy. It is suggested that neural innervation is important for the increased synthesis of vascular non-collagenous protein during the early hypertensive phase in the SHR.

Significance to Biomedical Research and Institute Program: This research project has revealed several significant points:

1. Sympathetic nerves may have a trophic effect on smooth muscle synthesis in small arteries.

2. An increase in non-collagen protein synthesis may be one of the primary pathogenic factors in spontaneous hypertension.

3. Hydralazine may not be a good long term therapy for hypertension since it does not appear to prevent the hypertrophy of the vessels.

Proposed Course of Project: These studies will be expanded to include the effects of all the major anti-hypertensive agents on the increased vascular protein synthesis in the hypertensive rat model. We are particularly interested in the effects of catecholamine receptor blocking agents on the non-collagen protein synthesis.

ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

The research program of the Molecular Disease Branch is directed toward the elucidation of the molecular mechanism involved in lipoprotein biosynthesis, transport, and metabolism. Current studies involve the systematic investigation of the enzymatic regulation of cholesterol biosynthesis, chemistry and metabolic turnover of plasma lipoproteins, enzymatic degradation of plasma triglycerides, and the quantitation and interrelationships of the apolipoproteins which function as structural components of plasma lipoproteins and cofactors for the enzymatic degradations of the constitutive lipids. A detailed knowledge of lipoprotein biochemistry and metabolism is a prerequisite to our understanding of the pathogenetic mechanisms involved in patients with disorders of lipid metabolism and atherosclerosis.

During the last year a detailed analysis of the enzymatic regulation of cholesterol biosynthesis has been initiated. These studies have been directed toward two specific areas. The first has involved the isolation and characterization of HMG-CoA reductase, the major rate-limiting enzyme in the cholesterol biosynthetic pathway. This enzyme has been purified to homogeneity, and a number of chemical properties of the isolated enzyme determined. A detailed analysis of the molecular properties of the enzyme, and the modes of enzyme regulation including allostearic, covalent modification and enzyme synthesis are under investigation. A second series of studies on regulation of cholesterol synthesis have been performed utilizing skin fibroblast cells grown in tissue culture. Cell cultures from normal individuals and a variety of patients with disorders of lipid metabolism have previously been established in the Branch. Studies on the role of plasma lipoproteins, polypeptide hormones and other agents which regulate cholesterol biosynthesis are under investigation and will greatly facilitate our understanding of lipid biosynthesis in normal individuals, and patients with a variety of lipid disorders and atherosclerosis.

Prerequisite to our understanding of plasma lipid transport is a detailed knowledge of the chemistry and structure of the plasma apolipoproteins. During the last few years extensive investigation has focused on the isolation, characterization, and sequence analysis of human plasma apolipoproteins. To date at least six different apoproteins have been extensively characterized with the amino acid sequences of several of the A and C apolipoproteins being completed. Current efforts are directed toward a comprehensive analysis of the arginine-rich apolipoprotein, apoLP-D, C-II and the polymorphic variants of A-I. Additional studies have been undertaken on a comparison of the amino acid sequences of apolipoproteins from normals and patients with a variety of hyperlipidemias.

Of particular importance over the last year has been the recognition that several of the human apolipoproteins undergo reversible self-association. Detailed analysis of the molecular properties of A-I, A-II, reduced and carboxymethylated A-II and C-I have indicated that the molecular structure of

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each apoprotein is a sensitive function of solvent composition, pH and protein concentration. A-II, for example, at low protein concentration is monomeric and resembles a random coil in aqueous solutions. With self-association A-II forms a discrete dimer with a marked increase in secondary and tertiary structure. Comparable results were obtained with the C-I apoprotein which self-associates with an equilibrium best characterized as a monomer-dimer-trimer. The association of monomeric C-I into an oligomeric complex is characterized by a major shift in conformation from a random coil into an oligomer with a markedly increased molecular structure. To our knowledge, C-I undergoes the greatest change in conformation with association thus far reported in the literature.

The recognition that plasma apolipoproteins self-associate is of major importance in our interpretation of ligand binding, protein-protein and protein-lipid interactions. In the past, the increase in conformation observed with recombination of apoproteins and lipids was used as an index of reassociation of the lipoprotein complex. Extensive studies on isolated apoproteins, peptide fragments, and synthetic peptides have utilized this criterion for reassembly. The realization that apoproteins self-associate will necessitate a reevaluation of the previously reported recombination experiments. It is therefore apparent that a knowledge of the molecular properties of each of the apoproteins is a fundamental requirement for the interpretation of ligand binding, protein-protein, and protein-lipid interactions. The information acquired from these studies will permit a greater appreciation of the nature of the forces involved in these interactions, and will also permit a more detailed investigation into the specific amino acid residues which are involved in lipid-protein interactions.

In last year's annual report we described studies designed to evaluate the quaternary structure of the intact high density lipoproteins. These studies utilized a variety of physical techniques including ^{13}C and ^{31}P nuclear magnetic resonance, circular dichroism and fluorescence to evaluate the molecular organization of the lipid and protein in the lipoprotein particles. These studies suggested that HDL was a spherical micelle (ca. 100 Å in diameter) with the protein imbedded in the lipid rather than covering the surface of the micellar particle. These studies have been extended during the last year to a detailed study of low density lipoproteins (LDL). Using the variety of techniques discussed in last year's report, we have demonstrated that LDL, like HDL, appears to be a spherical micelle with the polar head groups of the phospholipids readily exposed to the aqueous solvent. It appears, therefore, that the quaternary structure of both LDL and HDL are similar, with the phospholipids organized in a micellar structure and the protein intercalated between the phospholipid molecules.

During the last year the Branch has continued previous studies on the isolation and characterization of the apolipoproteins and lipoproteins in a variety of disorders of lipid metabolism. Of particular interest has been the continued study of patients with Tangier disease. Additional analysis of patients with this disease have extended previous studies, and demonstrated triglyceride enrichment of LDL, even in those patients with normal triglyceride levels. These studies suggest that normal HDL is required for the generation of chemically normal LDL. Studies on the exchange of C apoproteins between the

VLDL and HDL density regions were evaluated following VLDL triglyceride hydrolysis after heparin injection. In contrast to findings in normals and hypertriglyceridemic subjects, lipolysis did not lead to observable net transfer of C-apolipoproteins into the HDL density region. This study implies that LpC derived from VLDL was not capable of forming a complex that had a hydrated density comparable to native HDL. Thus, normal HDL may be necessary for C apolipoprotein transfer from VLDL.

A major area of fruitful research over the years in the MDB has been the systematic kinetic analysis of lipoprotein turnover studies. These studies have elucidated the LDL catabolic defect in familial hypercholesterolemia, the VLDL-LDL precursor product relationship, C apolipoprotein exchange between VLDL and HDL, and the coincident metabolism of the two major apolipoproteins, A-I and A-II, of HDL. Current studies have included the kinetic analysis of the VLDL and HDL_2 elevations produced by estrogen administration, the determinations of increased synthesis, as well as paired LDL catabolism in a patient with homozygous familial hypercholesterolemia, and the delineation of an HDL catabolic defect in heterozygous patients with Tangier disease. Future investigations in this area will include simultaneous turnover studies of two different density fractions in patients with a variety of lipid disorders, including familial hypercholesterolemia, and homozygous Tangier disease. Refinement of current turnover research techniques will include the study of the metabolism of individual apoproteins including C-I, C-II, C-III, apoD and the arginine-rich apoproteins.

Adaptation of immunoassay techniques to the measurement of individual apoproteins in plasma has recently been undertaken in the Branch. The radioimmunoassay system for A-I has been established and applied to a variety of studies in normals and patients with lipoprotein abnormalities. Of particular interest was the observation that the A-I levels in Tangier homozygotes were 1% of normal. A 50% reduction in A-I levels was observed in Tangier heterozygotes and patients with type V phenotype. The development of specific quantitative techniques for the measurement of specific apolipoproteins will markedly facilitate our studies related to lipoprotein turnover and metabolism.

The Type II Coronary Intervention Study, designed to test the hypothesis that reducing the plasma LDL levels will favorably affect the progress of established coronary artery disease, is now going into its fifth year. During the last year recruitment has been terminated due to the slow acquisition of protocol-acceptable patients, the unexpected low incidence of patient death and myocardial infarction, and the question of reliability of angiography as an end point. In the ensuing months patients already randomized will be followed as outlined in the initial protocol. In addition a detailed study of the angiogram and its reliability as a useful criterion of cardiac vascular disease will be investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02002-03 MDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
NHLI Type II Coronary Intervention Study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert I. Levy, M.D., Director, NHLI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLI
John F. Brensike, M.D.
Irving K. Loh, M.D.
Robert Aldrich, M.D.
James Battaglini, M.D.
Beverly Rogers, R.N.
Marjorie Myrianthopoulos, Dietitian

COOPERATING UNITS (if any) Cardiology Branch, NHLI; Lipid Metabolism Branch, NHLI; Radiology Department, NIH; Pulmonary Branch, NHLI; Clinical Center Administration, NIH; Data Management Branch, Division of Computer Research and Technology, NIH

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry Section

INSTITUTE AND LOCATION
NHLI - Building 10, Room 6D-15

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
13	6.5	6.5

SUMMARY OF WORK (200 words or less - underline keywords)

This study may answer or shed considerable light on three questions of great interest to the Institute and to all those involved in heart research.

1. Is angiography a reliable and useful tool for the evaluation of coronary artery disease? Can changes on angiography be read reliably enough to serve as an endpoint for scientific studies or important clinical decisions?

2. Can we significantly decrease the incidence of M.I. or death without any additional new treatments or medications?

3. Will lowering cholesterol slow down, stop, or reverse the progression of coronary artery disease?

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Project Description:

Objective:

The primary aim of this study was to determine whether lowering the LDL cholesterol in patients with premature coronary artery disease and type II will slow, stop, or reverse the progression of coronary artery disease (i.e., to test the lipid hypothesis in a specific group of high risk patients).

Methods Employed:

Two hundred-fifty patients with coronary artery disease demonstrated by coronary angiography, who met all the criteria outlined in the protocol, were to be randomly split into two equal groups (treatment - 24 grams cholestyramine and diet control - control - 24 grams placebo and diet control). They would be followed in a double blind fashion monthly for two to five years. Repeat coronary angiography was to be performed at two years and at five years.

The end points were to be: (1) a significant difference in the progression of coronary disease as shown on angiography, or (2) a significant difference in new myocardial infarctions or death, or (3) lack of any of the above at five years.

Because (1) the rate of recruitment has been so slow, (2) the patients have done much better than expected, (3) the data analysis of the information obtained has been very difficult, and (4) the reliability of the endpoint (angiography) has been questioned, it was decided to stop recruitment and to evaluate the reliability of the endpoint with a separate study. If the endpoint is reliable, then continued in-depth studies on the current patients including repeat angiography at a four-to-five year time interval are in order to see why they are doing so well.

Major Findings:

1) Patients in the program are dying and having heart attacks at a much lower rate than outside groups, even after correcting for severity of coronary disease on angiography, ventricular disease, age and sex.

2) Patients in the program are dying and having heart attacks at a much lower rate than patients with similar indications of coronary disease who normalized on the diet and were discharged from the program for this reason.

3) The reliability of the angiogram as a scientific tool for measuring progression of coronary disease has been questioned. If it is reliable (this is being checked), our results show: a) much less progression of CAD in study patients at two years than in any other patient group reported upon; b) regression in some patients (10%) - previously unreported except 2-3 individual cases mentioned within other papers.

4) An additional year's experience has confirmed all of the preliminary observations made last year (see previous year's report) and negated none of them.

Significance to Biomedical Research and the Program of the Institute:

This study may answer or shed considerable light on three questions of great interest to the Institute and to all those involved in heart research.

1) Is angiography a reliable and useful tool for the evaluation of coronary artery disease? Can changes on angiography be read reliably enough to serve as an endpoint for scientific studies or important clinical decisions?

2) Can we significantly decrease the incidence of M.I. or death without any additional new treatments or medications?

3) Will lowering cholesterol slow down, stop or reverse the progression of coronary artery disease?

Proposed Course:

All of our efforts over the next three to six months are going into the detailed study of the angiogram and its reliability and into the conversion of our previous data into a new computer system which should make analysis and patient care monitoring better and easier. Follow-up of already randomized subjects will continue. All other activities have been suspended indefinitely.

Publications:

1. Borer, J. S., Brensike, J. F., Redwood, D. R., Itscoitz, S. B., Passamani, E. R., Stone, N. J., Richardson, J. M., Levy, R. I., and Epstein, S. E.: Limitations of the electrocardiographic response to exercise in predicting coronary artery disease. New Eng. J. Med. 293: 367, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02003-05 MDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Biochemistry and Metabolism of Plasma Lipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Ernst J. Schaefer, M.D., Staff Associate, Molecular Disease Branch, NHLI
Robert I. Levy, M.D., Director, NHLI
H. Bryan Brewer, Jr., M.D., Chief, Molecular Disease Branch, NHLI
Leslie L. Jenkins, M.A.

COOPERATING UNITS (if any) Mones Berman, Ph.D., Lab. Theoretical Biology, NCI; Robert Goebel, M.D., Lab. Theoretical Biology, NCI; Shlomo Eisenberg, M.D., Hadassah Medical Center, Jerusalem, Israel; Frank T. Lindgren, Ph.D., Univ. California, Berkeley, California

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry Section

INSTITUTE AND LOCATION
NHLI - Building 10, Room 7N-117

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.5	2.0

SUMMARY OF WORK (200 words or less - underline keywords)

Understanding the effect of estrogens on plasma lipoproteins may help to explain the observed lipoprotein differences between males and females, and may help to elucidate the markedly disparate age matched incidence of atherosclerosis between males and females in our population. Premenopausal females have significantly lower VLDL levels and significantly higher HDL_2 levels than do males. The higher HDL_2 levels can be explained by an estrogen effect. The lower VLDL levels are presumably due to progesterone, the other major ovarian hormone.

Further understanding of VLDL and HDL metabolism in normal and dyslipoproteinemic subjects (such as patients with Tangier disease) may help to elucidate the role of these lipoproteins in free cholesterol and cholesteryl ester metabolism.

A further definition of the defect in familial type IIa hyperlipoproteinemia will help us to understand regulation of cholesterol plasma levels, and will complement LDL receptor studies which have been carried out in the fibroblasts of these patients.

848

PHS-6040
(12-75)

Project Description:

Objectives:

1) To elucidate the metabolic behavior of HDL_2 and HDL_3, subfractions of HDL, extending the already developed multicompartmental model of HDL metabolism.

2) To study the effects of estrogens on VLDL, LDL, and HDL.

3) To study VLDL, LDL, and HDL metabolism in normal and dyslipoproteinemic subjects before and during various diet and drug perturbations.

4) To establish methodology for studying the metabolism of individual apoproteins as well as lipoproteins.

Methods Employed:

The isolation, purification and iodination of VLDL, LDL and HDL have been described in previous annual reports. In addition to using SDS gels for apoprotein separation, we have adopted newer gel electrophoresis methodology utilizing tetramethyl urea, which selectively precipitates the B peptide and delipidates the lipoprotein prior to electrophoresis. This method allows for rapid apoprotein content analysis using gel scanning techniques. Methodology for performing combined ^{131}I and ^{125}I lipoprotein turnover studies has been adopted so that one can follow the metabolism of two lipoprotein classes at any one time. ^{131}I lipoprotein studies are currently being monitored with the whole-body counter, as well as plasma and urine collection, adding another technique for measuring extravascular lipoprotein turnover.

Major Findings:

Previous work from this laboratory has shown:

1) The major defect in patients who are heterozygotes for familial type IIa hyperlipoproteinemia is decreased LDL catabolism.

2) The VLDL, LDL apoprotein B precursor product relationship.

3) The C apoprotein exchange between VLDL and HDL.

4) The homogeneous metabolism of the apoproteins A-I and A-II, the major apoproteins of HDL.

By July 1, 1976 thirteen lipoprotein turnover studies will have been completed. Six studies have been performed in three normal female subjects before and during estrogen administration. Four studies have been completed in patients who are both heterozygous (2) and homozygous (2) for Tangier disease (HDL deficiency). The Tangier homozygotes received ^{125}I HDL from a

normal volunteer and simultaneously received autologous ^{131}I VLDL. The same iodinated HDL that the Tangier homozygotes received was given to a separate normal volunteer who simultaneously received autologous ^{131}I HDL. A second study will be performed on this normal volunteer examining HDL_2 and HDL_3 metabolism. In addition, we have studied VLDL and LDL metabolism in one patient with homozygous familial type IIa hyperlipoproteinemia, as part of an on-going project to examine lipoprotein metabolism in this condition.

These studies have revealed the following insights:

1) Estrogen administration causes elevation of plasma VLDL and HDL_2 levels.

2) HDL catabolism is enhanced in patients with Tangier disease, and this catabolism is through an intravascular pathway.

3) Study of one patient at the NIH and studies elsewhere (five patients) indicate that type IIa homozygotes have some increased LDL synthesis as well as marked impairment of LDL catabolism.

Significance to Biomedical Research and the Program of the Institute:

Understanding the effect of estrogens on plasma lipoproteins may help to explain the well-known lipoprotein differences between males and females, and may help to elucidate the markedly disparate age matched incidence of atherosclerosis between males and females in our population. Premenopausal females have significantly lower VLDL levels and significantly higher HDL_2 levels than do males. The higher HDL_2 levels can be explained by an estrogen effect. The lower VLDL levels are presumably due to progesterone, the other major ovarian hormone.

Further understanding of VLDL and HDL metabolism in normal and dyslipoproteinemic subjects (such as patients with Tangier disease) may help to elucidate the role of these lipoproteins in free cholesterol and cholesteryl ester metabolism.

A further definition of the defect in familial type IIa hyperlipoproteinemia will help us to understand regulation of cholesterol plasma levels, and will complement LDL receptor studies which have been carried out in the fibroblasts of these patients.

Proposed Course:

Continuing objectives include further definition of lipoprotein metabolism in normal and dyslipoproteinemic subjects in baseline states and during diet and drug perturbations. Other objectives are to study the metabolism of the individual apoproteins C-I, C-II, C-III and the arginine-rich peptide, apoproteins involved in VLDL and HDL metabolism.

Publications:

1. Levy, R. I., Blum, C. B., and Schaefer, E. J.: The composition, structure and metabolism of high density lipoprotein. In Greten, H. (Ed.): Lipoprotein Metabolism, Heidelberg, Springer-Verlag, 1976, p. 56.

2. Blum, C. B., and Levy, R. I.: Interconversions of apolipoprotein fragments. Ann. Rev. Med. 26: 365, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02009-08 MDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure and Function of Polypeptides and Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

H. Bryan Brewer, Jr., M.D., Chief, Molecular Disease Branch, NHLI
Thomas Fairwell, Ph.D.
Rosemary Ronan, B.A.
Thomas Bronzert, B.S.
Ann LaRue, B.A.
Anne Houser, B.S.

COOPERATING UNITS (if any)

Claude Arnaud, Mayo Clinic, Rochester, Minnesota

LAB/BRANCH
Molecular Disease Branch

SECTION
Section on Peptide Chemistry

INSTITUTE AND LOCATION
NHLI - Building 10, Room 7N-117

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.0	2.5

SUMMARY OF WORK (200 words or less - underline keywords)

This work is directed toward the development of improved methodologies for the sequence analysis of polypeptides and proteins of biological interest. Of particular interest are the sequence analysis of proteins and enzymes involved in lipid transport and cholesterol biosynthesis. In addition, this work is directed toward the elucidation of the structure, function, and physiological role of polypeptides in cellular metabolism.

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Project Description:

1) Objective:

a) Isolation, characterization, and sequence analysis of human parathyroid hormone (HPTH).

b) Isolation and characterization of peptide fragments of HPTH for use as selective reagents for the immunoassay of HPTH in clinical disorders of calcium metabolism and metabolic bone disease.

Methods Employed:

HPTH has been isolated from parathyroid adenomas from patients undergoing surgery for primary, secondary or tertiary hyperparathyroidism in this country and abroad. The methods of isolation and characterization have been detailed in previous annual reports and include the extraction of defatted adenomas by 8 M urea in 0.2 M hydrochloric acid and fractionation with ether, acetic acid, sodium chloride and trichloroacetic acid. The resulting TCA powder is further purified by gel filtration and ion exchange chromatography.

Major Findings:

a) HPTH has been isolated in homogeneous form and characterized as outlined in previous reports. Previous studies of HPTH have included the determination of the amino acid sequence of the biologically active region of HPTH, the analysis of the molecular properties of the intact hormone, and the corresponding biologically active synthetic fragment from a variety of species. Recent studies have continued on the collection of additional hormone from glands supplied by several hundred laboratories around the world. Despite the relative minute amount of homogeneous material available, we have been able to develop procedures for the isolation and characterization of peptide fragments of HPTH derived from tryptic digestion, and selective cleavage of the arginine residues following citroconylation of the lysine residues. Analysis of these peptide fragments has permitted the development of a preliminary model for the structure of HPTH. These peptides are currently being purified in a preparative scale which will permit the determination of the complete structure of HPTH.

b) The isolation and characterization of peptide fragments of HPTH has permitted the development of specific reagents for use in the immunoassay. Studies outlined in previous annual reports have indicated that the major form of circulating parathyroid hormone in hyperparathyroid plasma is a carboxyl terminal fragment. Isolation and characterization of a 53-84 carboxyl terminal fragment permitted the characterization of several antisera used in the clinical assays for human plasma, and the categorization of these assays into amino and carboxyl antisera. A recent exciting observation in these studies has been the detection by Dr. Claude Arnaud of a large number of carboxyl terminal fragments in the 7.5% TCA soluble fraction obtained during

the initial extraction of HPTH. This fraction was previously discarded since it contained no biologically active hormone. Identification of the carboxyl terminal fragments in this fraction, and immunization of goats with this material resulted in the production of antisera to the carboxyl terminal region of HPTH. The antibodies produced in this manner have been characterized by the 53-84 fragment and shown to be specific for the carboxyl terminal region. The availability of these fragments, and immunoassay should permit the widespread use of a specific carboxyl assay for HPTH in clinical disorders of calcium metabolism.

2) Objective:

a) Development of an automated high speed liquid chromatographic method for the identification and quantitation of phenylthiohydantoin amino acids (PTH amino acids) obtained from the manual and automated degradations of polypeptides and proteins.

b) Development of a rapid qualitative technique for the identification of PTH arginine and histidine from the aqueous layer of the Edman degradation.

Methods Employed:

PTH amino acids were chromatographed in a Dupont 830 liquid chromatograph equipped with an 833 programmable gradient, 836 multiple wavelength detector, 833 flow controller, and 838 automatic sampler.

Thin layer chromatographic analysis of PTH arginine and histidine was performed on Cheng-Chin polyamide plates developed with n-butylacetate: methanol containing butyl-PBD as a fluorescent indicator.

Major Findings:

a) Complete resolution (98%) of all PTH amino acids has been achieved with two coupled 4.6 mm x 25 cm Zorbax-ODS columns utilizing a curvilinear gradient of 10 to 42% acetonitrile in 0.01 N sodium acetate, pH 5.0. The eluates were monitored at 254 and 320 nm, the latter wavelength permitting the identification of dehydro-serine and dehydro-threonine. These derivatives have been particularly difficult to identify by other methods of analysis. Application of this technique to manual and automated Edman degradations of peptides and proteins has permitted the identification of 27 Edman derivatives per day. The development of a high speed liquid chromatographic technique offers several distinct advantages to other techniques, including the quantitation of all derivatives at the nanogram level, and the differentiation of aspartic-asparagine, glutamic-glutamine, and lysine. The latter derivatives are particularly difficult to identify due to selective absorption and thermal decomposition of the derivatives during gas chromatographic analysis.

b) A TLC method for the qualitative identification of histidine and arginine has been developed which permits the rapid identification of these

derivatives from the aqueous layer of the Edman degradation. This technique involves the use of Cheng-Chin polyamide plates containing a fluorescent indicator. This method permits the detection of histidine and arginine at the nmole level. These two derivatives are poorly identified by other techniques, including gas chromatography and mass spectroscopy.

The development of both the high speed liquid chromatographic system and the TLC methodology now permits the identification of each amino acid residue in a sequence by a variety of different techniques including gas-liquid, thin-layer and high-speed liquid chromatography, and mass spectroscopy. All of these techniques are required to adequately document each residue in a specific amino acid sequence due to the specific limitation of each methodology, and the selective destruction or adsorption of particular amino acid derivatives with each technique. The culmination of the development of each of these techniques will also permit the detailed analysis of the stability and chromatographic properties of each of the PTH amino acids, thereby permitting the improved accuracy of identification of specific amino acid residues in the structural analysis of proteins and polypeptides.

3) Objective:

To develop improved automated methods for the sequence determination of peptides and proteins.

Methods Employed:

Over the last few years there has become an increasing demand for the development of improved methodology for the sequence analysis of small quantities of proteins and the degradation of large proteins (> 200 residues). A variety of biologically important proteins including interferon, transplantation, antigens, polypeptide hormones, apolipoproteins and enzymes can be obtained in only relatively minute amounts. Alternatively, large molecular weight proteins such as apobetalipoprotein, immunoglobulin, and enzymes require significant amounts of protein for automated sequence analyses in order to obtain sufficient nanomoles of each PTH amino acid for identification.

A variety of new techniques are under development to address these problems. These include the development of improved programs and new spinning cups for the automated Beckman sequencer, and the attachment of small peptides (< 30 residues) to solid supports.

Major Findings:

A variety of new volatile peptide programs have been evaluated and a current program utilizing a dimethylallylamine buffer with selected periods of nitrogen drying has been developed. These programs permitted the complete sequence analysis in a single run of both a 25 and 32 amino acid cyanogen bromide peptide of human apoA-I.

Improved techniques have been developed for the attachment of peptides to

solid supports including glass beads. Specific methods of attachment that seem the most meritorious at the present time include the attachment of cyanogen bromide peptides via the carboxyl terminal homoserine residue, and the use of diphenylisothiocyanate. Peptides attached to glass beads have now been found in several sequence runs to adequately degradate in the spinning cup, with no major loss of beads from the cup.

Significance to Biomedical Research and the Programoof the Institute:

This work is directed toward the development of improved methodologies for the sequence analysis of polypeptides and proteins of biological interest. Of particular interest are the sequence analysis of proteins and enzymes involved in lipid transport and cholesterol biosynthesis. In addition, this work is directed toward the elucidation of the structure, function, and physiological role of polypeptides in cellular metabolism.

Proposed Course:

During the next year a systematic investigation of new techniques for the sequence analysis of proteins on a minute as well as large scale will be continued. In addition, it is planned to complete the covalent structure of human parathyroid hormone. The completion of the sequence of HPTH will permit the synthesis of selected regions of the hormone which will be of importance for the clinical development of additional immunoassays for specific regions of the human hormone. The ultimate development of a variety of different immunoassays of HPTH will permit a more extensive analysis of the peripheral metabolism and circulating fragments of PTH. These studies should improve our understanding of the structure-function requirements for biological activity and the mode of regulation of hormonal activity.

Publications:

1. Arnaud, C. D., and Brewer, H. B., Jr.: Parathyroid hormone: structure and immunoheterogeneity. In Kuhlencordt, F., and Kruse, H. P. (Eds.): Calcium Metabolism, Bone and Metabolic Bone Diseases, New York, Springer-Verlag, 1975, p. 295.

2. Brewer, H. B., Jr., Fairwell, T., Ronan, R., and Arnaud, C. D.: Recent studies on human parathyroid hormone. Clinical Endocrinology, in press.

3. Arnaud, C. D., Dibella, F. P., Brewer, H. B., Jr., Zawistowski, K., and Verheyden, J.: Human parathyroid hormone: biologic and immunologic activities of its synthetic (1-34) tetratriacontrapeptide and the utility of a carboxyl-terminal specific radioimmunoassay in assessment of hyperparathyroid syndromes. In Talmage, R. V., Owen, M., and Parsons, J. A. (Eds.): Calcium Regulating Hormones, New York, Excerpta Medica, 1975, p. 15.

4. Brewer, H. B., Jr., Fairwell, T., Ronan, R., Rittel, W., and Arnaud, C.: Human parathyroid hormone. In Talmage, R. V., Owen, M., and Parsons, J.A.

(Eds.): Calcium Regulating Hormones, New York, Excerpta Medica, 1975, p. 23.

5. Bronzert, T., and Brewer, H. B., Jr.: Automated high speed liquid chromatographic separation of the amino acid phenylthiohydantoins (PTH amino acids) obtained from the Edman degradation of peptides and proteins. J. Chromatography, in press.

6. Bose, S., and Brewer, H. B., Jr.: An improved system for the separation and identification of phenylthiohydantoin derivatives of histidine and arginine by thin-layer chromatography. Anal. Biochem. 71: 42, 1976.

7. Appella, E., Tanigaki, N., Fairwell, T., and Pressman, D.: Partial amino acid sequences of the heavy chains of human HLA histocompatibility antigens. Biochem. Biophys. Res. Commun., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02010-05 MDB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Structure and Function of the Plasma Lipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

H. Bryan Brewer, Jr., M.D., Chief, Molecular Disease Branch, NHLI
Thomas Fairwell, Ph.D.
James C. Osborne, Jr., Ph.D.
Ann LaRue, B.A.
Anne Houser, B.S.
Rosemary Ronan, B.A.
Thomas Bronzert, B.S.

COOPERATING UNITS (if any) John Gwynne, M.D., Univ. North Carolina, Chapel Hill, N.C.; Edward Sokoloski, B.S., Chemistry Branch, NHLI; Robert Highet, Ph.D., Chemistry Branch, NHLI; Robert Mahley, M.D., Lab. Exptl. Atherosclerosis, NHLI

LAB/BRANCH
Molecular Disease Branch

SECTION
Section on Peptide Chemistry

INSTITUTE AND LOCATION
NHLI, Building 10, Room 7N-117

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	1.5	3.00

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are directed toward the elucidation of the molecular structure of the plasma lipoproteins, and the role that primary structure plays in lipid-protein and protein-protein interactions. In addition, the role of plasma lipoproteins as cofactors for enzymes, precursors for steroidogenesis, vehicles for lipid transport, and regulators of cholesterol biosynthesis are systematically being investigated. A detailed knowledge of the chemistry of the plasma lipoproteins is a prerequisite to our understanding of lipid transport in normal and dyslipoproteinemic subjects.

858

PHS-6040
(12-75)

Project Description:

1) Objective:

Isolation and characterization of the polymorphic forms of apoA-I from human high density lipoproteins (HDL).

Methods Employed:

Human HDL was isolated by preparative ultracentrifugation between densities 1.063 and 1.21 g/ml. The isolated density fraction was delipidated with chloroform methanol and fractionated by Sephadex G-200 and DEAE chromatography. Purity of the isolated apoproteins was determined by disc gel electrophoresis in 8 M urea and sodium dodecyl sulfate. Molecular weight determinations were performed with the Beckman model E ultracentrifuge, and secondary structure was assessed by circular dichroism.

Major Findings:

Two specific polymorphic forms of apoA-I have been isolated in homogeneous forms following DEAE chromatography. The two forms of apoA-I have been shown to be identical in amino acid composition following acid hydrolysis, and amino terminal sequence. The two polymorphic forms are readily separated on disc gel electrophoresis at alkaline pH, however, are identical in migration at acid pH and following SDS electrophoresis. The two components are of similar molecular weight when analyzed by analytical ultracentrifugation. The secondary structure (α helix) of the two forms was also identical. Cyanogen bromide cleavage of the two polymorphic forms revealed four major peptides which appear to be identical in composition and structure. Therefore, no consistent difference between the two molecular forms of apoA-I has been demonstrated by amino acid composition, cyanogen bromide fragmentation, molecular weight determination or protein association. Additional studies are underway to delineate the carbohydrate, lipid and amide content of the two polymorphic forms.

2) Objective:

Determination of the complete amino acid sequence of human apoA-I.

Methods Employed:

The methods of sequence analysis of apoA-I are detailed in previous annual reports, and include the isolation and characterization of peptide fragments generated from cyanogen bromide and trypsin cleavage of native and citroconylated apoA-I. Individual peptides were purified to homogeneity by a combination of gel filtration and ion exchange chromatography. The sequence of intact apoA-I and peptide fragments was determined by manual and automated Edman degradation. The phenylthiohydantoin amino acids were identified by thin-layer, gas-liquid and high speed liquid chromatography and mass spectroscopy.

Major Findings:

The amino acid sequence of apoA-I has essentially been completed. The molecule is a single chain polypeptide with no cystine residues. The structure contains no regions of linear hydrophilic residues. This is consistent with our previous sequence analysis of apoC-III and apoA-II which were devoid of linear hydrophobic sequences. Detailed analysis of the amino acid sequence of apoA-I reveals distinct areas of isostearic heterogeneity. Detailed studies will be required to determine the degree of structural polymorphism which exists in apoA-I in normal man.

3) Objective:

Comparison of the amino terminal sequence of human, rat, monkey, swine and dog A-I.

Methods Employed:

Similar to those outlined above and in previous annual reports.

Major Findings:

The major apoprotein that has thus far been isolated from HDL in a variety of different species has a molecular weight of approximately 28,000, a single amino terminus, and no cystine residues. These apoproteins appear to be analogues of human apoA-I. In contrast, apoA-II has been shown to exist as either a monomer or dimer, and to vary over a wide concentration range in different species of HDL. We, therefore, undertook in collaboration with Dr. Robert Mahley the isolation and characterization of apoA-I from dog, swine, and monkey HDL. The amino terminal sequence of each of the apoA-I proteins was determined on the automated Beckman sequencer as a probe for the homology of these apoproteins in the different species. The amino terminal sequences are as follows:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Human	Asp	Glu	Pro	Pro	Gln	Ser	Pro	Trp	Asp	Arg	Val	Lys	Asp	Leu	Ala	Thr	Val
Monkey	—	—	—	—	—	—	—	Thr	—	—	—	—	—	—	—	—	—
Swine	—	—	Asp	—	—	—	Gln	Ser	Pro	Trp	Asp	Arg	Val				
Dog	—	—	—	—	—	—	—	Gln	Ser	Pro	Trp	Asp	Arg	Val			

These results suggest that the major apoprotein in HDL from a variety of species is structurally similar, and comparable to the human apoA-I apoprotein. The specific role of apoA-I in HDL metabolism is as yet unknown. Its importance as a cofactor for the lecithin cholesterol acyl transferase enzyme, however, has been documented in various laboratories over the last few years.

4) Objective:

Isolation, characterization and sequence analysis of the C-apoproteins

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from normal HDL.

Methods Employed:

Similar to those outlined above and in previous annual reports.

Major Findings:

C-I, C-II, C-III$_1$ and C-III$_2$ have been isolated from normal HDL in homogeneous form. A sufficient quantity of apoproteins from normal plasma have been purified to permit a detailed comparison with the corresponding apoproteins isolated from hyperlipidemic plasma (principally type V patients). No detailed comparison of the C-apoproteins from hyperlipidemic and normals has as yet been published. In addition to the principal C proteins, another apoprotein has been identified and characterized. This apoprotein appears to be similar, if not identical, to the recently reported (McConathy, W., and Alaupovic, P., Biochem. 15: 515, 1976) LpD apoprotein. The availability of large quantities of C apoproteins from normal plasma will now permit the examination of the amino acid sequence and molecular properties of the individual apoproteins (see accompanying report).

5) Objective:

Determination of the molecular organization of the phospholipids in native high and low density lipoprotein (LDL).

Methods Employed:

The methods utilized to evaluate the spatial orientation of the phospholipids of HDL and LDL are detailed in previous annual reports. The principal techniques used in these studies is phosphorous nuclear magnetic resonance (^{31}P NMR) with the Fourier transform technique.

Major Findings:

Previous studies as outlined in last year's report have focused on the molecular organization of the phospholipids in HDL. These studies demonstrated that the polar head groups of the phospholipids were all exposed to the aqueous surface. The molecular structure of the phospholipids in HDL was therefore organized into a spherical micelle. We have further extended these studies to a detailed analysis of the molecular organization of the lipid in native LDL. LDL exhibits a characteristic ^{31}P NMR spectra containing two resonances separated by 6 ppm which correspond to phosphatidylcholine and sphingomyelin. The molecular structure of phospholipid in LDL was evaluated by titration with a variety of paramagnetic ions. Extensive studies were performed with the various reagents in order to ensure that no reagent precipitation occurs during the reaction. Titration with paramagnetic ions with model micelles and bilayers ha demonstrated a characteristic upfield shift of polar head groups exposed to the aqueous solvent. Phosphorous groups which are shielded due to their location on the inner surface of a bilayer are not shifted during

titration. This technique, therefore, permits the categorization of phospholipid particles into micelles or bilayers. Applications of this technique to the molecular structure of LDL has demonstrated a greater than 95% shift of polar heads following titration. Studies performed with different reagents gave similar results. These studies suggest that LDL, like HDL, is a spherical micelle with the majority, if not all, of the polar heads oriented toward the aqueous surface.

6) Objective:

Analysis of adrenal cholesterol uptake from plasma lipoproteins, and its regulation by ACTH.

Methods Employed:

Adrenal cholesterol uptake has been determined by incubating quartered glands obtained from normal and ACTH treated rats with purified HDL and LDL containing ^{14}C-4 or 1,2-^{3}H-cholesterol. Incubations were performed for up to 2 hours at 4°C and 37°C, in buffers containing aminoglutethimide to prevent conversion of cholesterol to pregnenolone. Incubations were performed in standard buffers and assayed for protein, cholesterol and radioactivity. Specific radiolipids were identified by thin-layer chromatography. Intracellular localization of labeled cholesterol was performed by differential centrifugations. Conversion of cholesterol to pregnenolone, the rate-limiting step in adrenal steroidogenesis, occurs in the mitochondria and was evaluated by the method of Mahaffee, et al., J. Biol. Chem. 249: 227, 1974.

Major Findings:

Adrenal glands from unstimulated or ACTH stimulated hypophysectomized rats selectively took up labeled cholesterol from HDL but not LDL. ACTH stimulation increased the transfer of cholesterol from HDL but not LDL. The process of cholesterol transfer from HDL was linear with time over two hours at 37°C, and greatly reduced at 4°C. The transfer progress was saturable, and the label showed a preferential location in specific subcellular fractions. Approximately 25% of the labeled cholesterol was recovered within the mitochondria fraction. The labeled cholesterol within isolated mitochondria could undergo conversion to pregnenolone. These studies suggest that rat adrenal tissue possesses an HDL specific, hormonally responsive mechanism for accumulating extracellular cholesterol. This HDL cholesterol appears to be the major precursor for adrenal steroidogenesis.

Significance to Biomedical Research and the Program of the Institute:

These studies are directed toward the elucidation of the molecular structure of the plasma lipoproteins, and the role that primary structure plays in lipid-protein and protein-protein interactions. In addition, the role of plasma lipoproteins as cofactors for enzymes, precursors for steroidogenesis, vehicles for lipid transport, and regulators of cholesterol biosynthesis are systematically being investigated. A detailed knowledge of the chemistry of

the plasma lipoproteins is a prerequisite to our understanding of lipid transport in normal and dyslipoproteinemic subjects.

Proposed Course:

We will continue our studies on the isolation, characterization, and sequence analysis of new human apoproteins, as well as apoproteins from a variety of other species. The determination of the structure of apolipoproteins continues to be a prerequisite for a detailed understanding of the structure and function of the plasma lipoproteins.

Publications:

1. Gwynne, J. T., Mahaffee, D., Brewer, H. B., Jr., and Wey, R. L.: Adrenal cholesterol uptake from plasma lipoproteins: regulation by ACTH. Proc. Natl. Acad. Sci., in press.

2. Mahley, R. W., Weisgraber, K. H., Innerarity, T., and Brewer, H. B., Jr.: Characterization of the plasma lipoproteins and apoproteins of the Erythrocebuspatas monkey. Biochem. 15: 1928, 1976.

3. Mahley, R. W., Weisgraber, K. H., Innerarity, T., Brewer, H. B., Jr., and Assmann, G.: Swine lipoproteins and atherosclerosis. Changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. Biochem. 14: 2817, 1975.

4. Gwynne, J., Palumbo, G., Brewer, H. B., Jr., and Edelhoch, H.: The interaction of apoA-I from human high density lipoproteins with lysolecithin. J. Biol. Chem. 250: 7300, 1975.

5. Gwynne, J., Palumbo, G., Osborne, J.C., Brewer, H. B., Jr., and Edelhoch, H.: The self-association of apoA-II, an apoprotein of human high density lipoprotein complexes. Arch. Biochem. Biophys. 170: 204, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02011-01

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Molecular Properties of Plasma Lipoproteins and Apolipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

James C. Osborne, Jr., Ph.D., Staff Associate, MDB, NHLI
H. Bryan Brewer, Jr., M.D., Chief, MDB, NHLI
Thomas Bronzert, B.S.
Ken Edwards, B.S.
Anne Houser, B.S.
Ann LaRue, B.A.
Rosemary Ronan, B.S.

COOPERATING UNITS (if any)

Harold Edelhoch, Ph.D., Clinical Endocrinology Branch, NIAMDD
Silvestro Formisano, Ph.D., Clinical Endocrinology Branch, NIAMDD

LAB/BRANCH
Molecular Disease Branch

SECTION
Section on Peptide Chemistry

INSTITUTE AND LOCATION
NHLI - Building 10, Room 7N-117

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.2	1.2	3.0

SUMMARY OF WORK (200 words or less - underline keywords)

This work is directed toward a greater understanding of the molecular structure and function of the plasma lipoproteins. An appreciation of the forces involved in lipid-protein and protein-protein interactions will be fundamental to our acquisition of knowledge related to the quaternary structure of lipoprotein particles. It is assumed that a fundamental understanding of the molecular forces and nature of the interactions between lipoprotein particles will be prerequisite to our understanding of lipid transport and metabolisms in normal individuals, and in patients with disorders of lipid metabolism and atherosclerosis.

PHS-6040
(12-75)

Project Description:

1) Objective:

In the metabolic conversion of VLDL to LDL the C-peptides, C-I, C-II and C-III, are released and exchanged with the corresponding peptides in HDL. The primary goal of this investigation is an evaluation of the mechanism of VLDL-HDL exchange and of the role of the C-peptides in the molecular organization of VLDL and HDL. Although the primary sequences of C-I and C-III have been published, very little is known about their properties in aqueous solution. Thus, the first phase of this study is an evaluation of the molecular properties of the isolated C-peptides as a function of protein concentration, solvent composition and temperature. A knowledge of these properties will provide a firm foundation for ligand binding and recombination studies and greatly simplify their interpretation.

Methods Employed:

Secondary structure, i.e., α-helical, β pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in fluorescence and absorption properties on a Perkin-Elmer model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular peptide interaction, was studied by molecular weight measurements on a Spinco Model E ultracentrifuge. Calculations and least square fits of the data to various models were made with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

ApoC-I, isolated from the human high density lipoprotein complex, was found to undergo self-association in aqueous solution. Moreover, major changes in secondary structure were found to accompany the association reaction. Thus, the protomers at neutral pH resembled random coils, whereas the oligomers were highly structured and resembled globular proteins. The association constant was found to decrease with decreasing pH and in the acid range the protomers became highly structured. These studies add apoC-I to the rapidly growing group of apolipoproteins that are known to self-associate in aqueous solution. The major conformational changes that accompany association make it clear that a knowledge of the molecular properties of apoproteins in aqueous solution is a fundamental requirement for the interpretation of ligand binding and recombination studies.

2) Objective:

Evaluation of the role of the major proteins, apoA-I and apoA-II, in the molecular organization of HDL. The first phase of this study, evaluation of the molecular properties of the isolated apoproteins, is nearing completion. It has been shown that apoA-I and apoA-II self-associate in aqueous solution and their molecular weights as a function of protein concentration have been

reported. The secondary and tertiary structure of apoA-II as a function of solvent composition and temperature is also now well-defined. The second phase of this study is an evaluation of the effects of various ligands (inorganic salts, lipids, other proteins, etc.) on secondary, tertiary and quaternary structure.

Methods Employed:

Those methods outlined in objective (1) above and the technique of equilibrium dialysis.

Major Findings:

Preliminary results indicated that the molecular properties of apoA-I, apoA-II and SCMC apoA-II were altered in the presence of calcium. The change in free energy, obtained by equilibrium dialysis, upon the binding of calcium was -3.9 Kcal/mole for apoA-I, -3.8 Kcal/mole for apoA-II and -3.8 Kcal/mole for SCMC apoA-II. The results also indicated that there were one, two and three binding sites for calcium on SCMC apoA-II, apoA-II and apoA-I, respectively. The binding of calcium favored the formation of oligomers for each of the apoproteins. Thus, the molecular weights at high calcium levels, approximately 50 mM, approached 17,000 for SCMC apoA-II, 32,000 for apoA-II and 95,000 for apoA-I. The ability to perturb the molecular properties of apoproteins with inorganic ions such as calcium could prove to be of great utility in the interpretation of recombination studies.

3) Objective:

It is now well-established that "hydrophobic" bonds play an important role in lipid-lipid, lipid-protein, and protein-protein interactions. However, although many investigators have addressed the properties of hydrophobic bonds, both experimentally and theoretically, our understanding of the phenomena has increased very little since the classical papers by Frank and Evans in 1945. We have, therefore, analyzed the thermodynamics of the self-association of SCMC apoA-II and data from other "hydrophobic" systems in the literature, such as micelle formation, in order to characterize more fully hydrophobic bond formation.

Major Findings:

The hydrophobic bond is usually characterized in the literature in terms of changes in entropy. If the reaction in question is entropy driven, that is, the entropy change upon association is positive, then the reaction is usually assumed to be hydrophobic in nature. However, an analysis of data from "hydrophobic" systems, such as the solubility of non-polar solutes, ligand bindings, protein-protein association, protein renaturation and micelle formation, indicates that the entropy and enthalphy of hydrophobic interaction are sensitive functions of temperature. Both thermodynamic parameters are positive at low temperatures and negative at high temperatures for hydrophobic bond formation. Thus, the sign of the entropy and enthalphy change for a

reaction is not a sufficient criteria for evaluating the "hydrophobic" contribution to the reaction scheme. Hydrophobic interactions are best characterized by the dependence of the enthalphy on temperature, i.e., changes in heat capacity. The results of this analysis reinforce the utility of calorimetric measurements on biological systems (the change in heat capacity can be directly measured) and should simplify the interpretation of hydrophobic interactions.

Significance to Biomedical Research and the Program of the Institute:

This work is directed toward a greater understanding of the molecular structure and function of the plasma lipoproteins. An appreciation of the forces involved in lipid-protein and protein-protein interactions will be fundamental to our acquisition of knowledge related to the quaternary structure of lipoprotein particles. It is assumed that a fundamental understanding of the molecular forces and nature of the interactions between lipoprotein particles will be prerequisite to our understanding of lipid transport and metabolisms in normal individuals, and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

During the next year it is planned to continue a detailed examination of the molecular structure of the plasma apoproteins including apoA-I and the C apoproteins. Additional studies on lipid-protein interactions will be continued with an emphasis on an understanding of the molecular forces involved in lipid binding.

Publications:

1. Osborne, J. C., Palumbo, G., Brewer, H. B., and Edelhoch, H.: The self-association of the reduced apoA-II apoprotein from the human high density lipoprotein complex. Biochemistry 14: 3741, 1975.

2. Edelhoch, H., and Osborne, J. C.: The thermodynamic basis of the stability of proteins, nucleic acids and membranes. Advan. Protein Chem. 30: 183, 1976.

3. Osborne, J. C., Palumbo, G., Brewer, H. B., and Edelhoch, H.: The thermodynamics of the self-association of the reduced and carboxymethylated form of apoA-II from the human high density lipoprotein complex. Biochemistry 15: 317, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02012-01

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Purification and Characterization of HMG-CoA Reductase and Its Role in the Regulation of Cholesterol Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Zafarul Beg, Ph.D., Visiting Scientist, MDB, NHLI
H. Bryan Brewer, Jr., M.D., Chief, Molecular Disease Branch, NHLI
John Stonik, B.S.

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry Section

INSTITUTE AND LOCATION
NHLI, Building 10, Room 7N-117

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	1.25	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

This work is directed toward a rapid purification procedure for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the major regulatory enzyme in hepatic cholesterol biosynthesis. Of special importance are the characterization and investigation of its regulatory role in the control of cholesterol biosynthesis.

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PHS-6040
(12-75)

Project Description:

1) Objective:

Isolation and characterization of hepatic HMG-CoA reductase.

Methods Employed:

Liver HMG-CoA reductase is bound to microsomal membranes. Microsomes were isolated by centrifugation (100,000 x g, 60 min.) of post-mitochondrial supernatant of freshly excised rat livers. The enzyme has been solubilized by slow freeze-thaw treatment. Buffer extraction of this microsomal preparation yielded a crude solubilized enzyme extract with high specific activity. The solubilized enzyme was further purified by a series of affinity gels. The purified enzyme has been characterized by regular, SDS and urea disc gel electrophoresis.

Major Findings:

HMG-CoA reductase has been isolated in homogeneous form by using a series of different affinity gels, including agarose-coenzyme A, agarose-NADPH, agarose-aminohexane, agarose-CAMP and agarose-ATP. The enzyme reversibly binds to these gels and could be eluted with a specific eluant.

The initial affinity gel which is currently in use is blue-dextran-sepharose. The soluble enzyme is passed through a column of blue-dextran-sepharose and reductase protein is eluted by 0.5 M KCl. The resulting preparation is further purified on an agarose-ATP column. The enzyme eluted off the ATP column is highly purified and has a specific activity of 800-1000 nmoles/min./mg protein. The purified enzyme on SDS gel electrophoresis shows only trace impurities; the subunit molecular weight for the enzyme measured by this technique is 65-70,000 (using bovine serum albumin as a standard).

The purified enzyme exhibits a major band on disc gel electrophoresis, with an apparent molecular weight of ∿200,000. The location of activity of the purified enzyme was established by analysis of 2 mm thick slices of gel. HMG-CoA reductase was eluted from the band containing enzymatic activity and rerun in an SDS system. The band containing enzymatic activity comigrated with the protein band assigned to purified HMG-CoA reductase. These SDS gel electrophoresis studies suggested that the purified enzyme is a trimer containing three identical subunits.

2) Objective:

Modulation of HMG-CoA reductase activity by ATP-Mg^{++} and cyclic AMP.

Methods Employed:

Unwashed microsomal HMG-CoA reductase can be inactivated by the addition of ATP-Mg^{++} in the incubation mixture. However, solubilized or purified

enzyme is not susceptible to ATP inactivation. Semi-purified enzymes can be inactivated by the addition of a protein fraction of liver cytosol precipitated at 30% ammonium sulfate saturation.

Major Findings:

Partially purified or purified HMG-CoA reductase has been shown to exist in two forms, i.e., inactive and active. The enzyme could be inactivated by the addition of 2 mM ATP-Mg^{++} and a cytosolic (30% cut) fraction which presumably contains protein kinase. This effect is augmented by low concentrations of cyclic AMP. The inactivated enzyme can be reactivated by the addition of 15 mM $MgCl_2$ and another cytosolic fraction (65-75% cut). The enzyme can be activated to various degrees by the addition of a 65-75% fraction. Little is known of the mechanisms by which reductase is activated and inactivated. The mechanism may be similar to that for regulation of acetyl-CoA carboxylase in which phosphorylation inactivates the enzyme and dephosphorylation activates it. This problem is currently under investigation.

Significance to Biomedical Research and the Program of the Institute:

These studies are directed toward an improved understanding of the control of cholesterol biosynthesis. An elucidation of the structure and mode of regulation of the major rate-limiting enzyme, HMG-CoA reductase, will permit a more detailed analysis of the factors regulating cellular cholesterol biosynthesis. These studies will ultimately provide additional information on lipid synthesis and transport in normal and dyslipoproteinemic subjects.

Proposed Course:

During the next year a systematic investigation of properties of HMG-CoA reductase will be continued. For this purpose, the enzyme will be isolated on a large scale using chicken livers and a continuous-flow centrifuge. In addition, it is planned to complete the physical characterization of rat liver HMG-CoA reductase. The short-term control of enzyme activity by phosphorylation-dephosphorylation will be evaluated with purified reductase protein using ^{32}P-ATP. These studies will expand our understanding of the structure-function requirements of this important enzyme, and also the mode(s) of its regulation in cholesterol metabolism.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02013-01

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Human Plasma Lipoproteins, Apolipoproteins and Lipolytic Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Peter N. Herbert, M.D., Head, Lipoprotein Structure Section, MDB, NHLI
Linda L. Bausserman, B.S.
Eve C. Church, M.S.
Donald S. Fredrickson, M.D.
Robert J. Heinen, B.S.
Lloyd O. Henderson, Ph.D.
Marguerite J. LaPiana, B.A.
J. Roger Lee, B.S.
Thomas A. Musliner, M.D.

COOPERATING UNITS (if any) R.M. Krauss, M.D., T.F. Forte, Ph.D., F. Lindgren, Ph.D., and A. Nichols, Ph.D., Donner Labs., Berkeley, Calif.; T.P. Bersot, M.D., Sect. Athero., NHLI; P.J. Dyck, M.D., Dept. Neurol., Mayo Clinic, Rochester, Minn.; R.D. Yee, M.D. and D.R. Bergsma, M.D., Clin. Br., NEI; J.J. Biemer, M.D., Dept.

LAB/BRANCH Path., St. Joseph's Hosp., Tampa, Fla., G. Assmann, M.D., Univ.Cologne, Cologne, W. Germany

SECTION
Molecular Disease Branch, Lipoprotein Structure Section

INSTITUTE AND LOCATION
NHLI, Building 10, Room 7N-117

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.8	2.3	3.5

SUMMARY OF WORK (200 words or less - underline keywords)

1) A sensitive and accurate radioimmunoassay system for the A-I apolipoprotein has been established and applied in studies of normals, subjects with Tangier disease and patients with a variety of hyperlipoproteinemias. ApoLP-A-I concentrations in Tangier patients were shown to be less than 1% of normal and reductions of 50% or greater were demonstrated in Tangier heterozygotes and patients with type V hyperlipoproteinemia. 2) Technical problems in the purification of apoLP-C-II have been resolved and sufficient quantities of the protein have been isolated to permit determination of its amino acid sequence and investigation of its structure-function relationships. 3) Characterization of the plasma lipoproteins and apolipoproteins of Tangier disease is almost complete. The data indicate that high density lipoproteins modulate the catabolism of very low density to low density lipoproteins. 4) Studies on the fractionation and characterization of the hepatic and extrahepatic triglyceride hydrolase enzymes have been initiated with the goal of purifying the enzymes and characterizing their substrate and cofactor specificities. 5) A preliminary survey of the plasma lipoproteins and apolipoproteins in type III hyperlipoproteinemia has been completed. Considerable variability in lipoprotein lipid and protein composition has been found among patients with presumably the same genetic defect.

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Project Description:

1) Objective:

Quantitative alterations in total plasma concentration and lipoprotein distribution of the apolipoproteins may be central to the pathophysiology of the hyperlipoproteinemias, hypolipoproteinemias and dyslipoproteinemias. This project is part of an effort to develop sensitive and accurate radioimmunoassay techniques for apolipoprotein quantitation.

Methods Employed:

Efforts to date have been devoted to the establishment of a radioimmunoassay for the A-I apolipoprotein. The apoprotein was isolated from normal high density lipoprotein by a combination of gel and ion-exchange chromatography. A monospecific antiserum was raised in sheep. ^{125}I labeled antigen was produced by a modification of the chloramine T procedure of Roth and co-workers (Methods in Enzymology, XXXVI, 225-228, 197) and separated from free iodine by two gel chromatographic steps. Conventional techniques using a double antibody system were employed. Data generated were analyzed using the computer program of Faden and Rodbard (National Technical Information Service, 3rd. edition, vols. 1 & 2, Springfield, Va., 1975).

Major Findings:

a) Normal plasma apoLP-A-I concentrations: greater than one hundred samples from normolipidemic individuals, males and females, in the second to sixth decade of life were analyzed. Average values for males were 97 mg/dl and 115 mg/dl for females, the sex difference being statistically significant ($p < 0.001$). The apoLP-A-I concentrations correlated positively ($R = 0.52$), but less strongly than anticipated with the plasma high density lipoprotein cholesterol level and a negative correlation with the total plasma cholesterol concentration was found ($R = -0.64$).

b) ApoLP-A-I concentrations in Tangier disease: Tangier disease (alpha lipoprotein deficiency) is characterized by the near total absence from the plasma of normal high density lipoproteins. Plasma from nine subjects homozygotic for this disorder were studied and plasma apoLP-A-I concentrations ranging from 0.2 to 3.4 mg/dl were found. The values in the majority of patients were less than 1% of those found in normal controls. Five obligate heterozygotic parents of these patients were tested and their apoLP-A-I concentrations ranged from 29-59 mg/dl, levels 50% or less of the normals.

c) Plasma apoLP-A-I levels in hyperlipoproteinemia: preliminary studies have been initiated to define the spectrum of apoLP-A-I concentrations in subjects with Fredrickson-Levy-Lees hyperlipoproteinemias, types I through V. Results to date indicated that subjects with even mild to severe hypertriglyceridemia do not have a selective depression of their apoLP-A-I concentration, suggesting that in many such subjects the HDL cholesterol concentrations may not adequately reflect the plasma A-protein content. Patients with type I

hyperlipoproteinemia may represent notable exceptions, since at least two have had values comparable to those found in the Tangier heterozygotes. In addition, subjects with severe hypercholesterolemia (often considered under the rubric "homozygous familial type II hyperlipoproteinemia") appear to have apoLP-A-I levels similar to those in Tangier heterozygotes and type I hyperlipoproteinemia.

2) Objective:

Determination of the covalent structure of apoLP-C-II.

Methods Employed:

Previously outlined in Z01 HL 02007-08 MDB.

Major Findings:

As detailed in last year's annual report, major problems have been encountered in obtaining sufficient apoLP-C-II to complete definition of its primary structure. These problems have related to inadequate dietary preparation of hypertriglyceridemic subjects prior to plasmapheresis, antibiotic resistant bacterial contamination of gel columns and contamination of several apoLP-C-II preparations with the sialic acid free form of apoLP-C-III. In addition, inadvertent oxidation of methionine residues in highly purified apoLP-C-II has greatly reduced yields from cyanogen bromide cleavages. The majority of these problems have now been circumvented and the primary structure analysis of apoLP-C-II should be completed in the next few months.

3) Objective:

Characterization of the plasma lipoproteins and apolipoproteins in Tangier disease.

Methods Employed:

The methods of lipoprotein isolation by isopycnic ultracentrifugation and apolipoprotein fractionation by gel and ion-exchange chromatography have been detailed in previous annual reports. In more recent studies the high density lipoproteins have been separated into two empirical classes (1.063-1.080 g/ml and 1.080-1.21 g/ml) to permit better resolution of large and small particles.

Major Findings:

a) The very low and low density lipoproteins have been studied in three additional subjects with Tangier disease including one 69 year old woman (in collaboration with Dr. Peter Dyck of the Mayo Clinic). This latter patient, newly discovered, is the oldest living subject with the disorder. The triglyceride enrichment of the low density lipoproteins noted in last year's

annual report has been confirmed in all subjects, including those with normal plasma triglyceride levels. These studies confirm the role of normal high density lipoproteins in the generation of chemically normal LDL.

b) Additional studies of the C-apolipoprotein content of Tangier VLDL has revealed that the previously noted selective and profound reduction in at least one female is not observed under all dietary conditions. This finding does not favor a sex-influenced C-apolipoprotein deficiency in this disorder.

c) An effort was made to determine whether the C-apolipoproteins could be transferred en masse to the high density flotation range in response to the induction of VLDL triglyceride hydrolysis after the injection of heparin. In contrast to findings in normals and hypertriglyceridemic subjects, lipolysis in the single patient studied did not lead to observable net transfer of C-apolipoproteins to the higher density class. This study implies that apoC in combination with lipid derived from VLDL is not capable of forming independent high density lipoproteins; and that transfer to normal high density lipoproteins is necessary to "conserve" the C-apolipoproteins in this density class.

d) The small quantities of HDL isolatable from Tangier plasma have been evaluated in terms of chemical composition, morphology, immunoreactivity and polyacrylamide gel patterns after total delipidation. Most of the lipid and protein found in this density class appears associated with a lipoprotein with the size and immunochemical properties of the Lp(a) lipoprotein. In the density 1.080-1.21 g/ml, however, the dominant species were spherical particles with an average diameter of 60 Å. These particles have been further purified by chromatography on agarose gel columns; they have alpha mobility on acrylamide gel electrophoresis, and contain apoLP-A-II but not apoLP-A-I. The significance of these particles in Tangier disease is the subject of current investigation.

4) Objective:

Determination of the substrate specificities of the plasma post-heparin triglyceride hydrolases.

Methods Employed:

Plasma was collected from normal subjects and patients with various forms of hyperlipidemia 10 minutes after the injection of heparin at a dose of 10 u/kg body weight. When large quantities of plasma were required for isolation of the hepatic and extra-hepatic enzymes by heparin affinity chromatography, plasma was obtained by plasmapheresis between 8 and 12 minutes after heparin injection. In some experiments the acetone-ether extracted enzyme from rat epididymal fat pads was employed. The techniques for enzyme assay employing ^{14}C-triolein and fatty acid titration when natural substrates are employed have been detailed in earlier annual reports.

Major Findings:

a) Severe hypertriglyceridemia is often associated with diminished plasma levels of both low and high density lipoproteins. Moreover, the LDL and HDL isolated from hypertriglyceridemic plasma is often strikingly enriched in triglyceride, presumably because of exchange of neutral lipid with chylomicrons and very low density lipoproteins. The possibility existed that the lipolytic enzymes of the capillary endothelium may utilize both the triglyceride-rich LDL and HDL as substrate. This hypothesis was tested in experiments employing either the total post-heparin lipolytic enzymes from normal subjects or the partially delipidated extract from rat epididymal fat pads. The triglyceride-rich LDL but not the HDL proved to serve as substrate for both enzyme systems. This finding may account for the hypobetalipoproteinemia of hypertriglyceridemia, but leaves unexplained the effects of elevations of the triglyceride-rich lipoproteins on HDL metabolism.

b) Earlier studies on the substrate specificity of the hepatic triglyceride hydrolase and lipoprotein lipase of extra-hepatic origin have been extended. The two enzymes have been isolated by batch elution of heparin affinity columns and stabilized in the partially purified form by albumin or plasma from an abetalipoproteinemic subject. The results confirmed studies where the two lipase activities were distinguished by difference after protamine inactivation of lipoprotein lipase. Chylomicrons proved to be the best substrate for the extra-hepatic enzyme, whereas very low density lipoprotein triglyceride was more activily hydrolyzed by the hepatic enzyme. Further characterization of the composition and apolipoprotein cofactor requirements of these enzymes are in progress.

c) Two siblings with severe hypertriglyceridemia manifested in the first decade of life with elevated plasma levels of both chylomicrons and very low density lipoproteins have been studied in detail. Post-heparin lipolytic activity was tested in both sibs. The propositus had low levels of both hepatic and extra-hepatic enzymes, but both were within the broad normal range. Her brother had normal levels of both enzymes. The postheparin enzymes of the propositus were fractionated by heparin affinity chromatography and the kinetics of their reaction against natural and artificial substrates were compared with normal controls. No gross differences were identified in preliminary experiments. The findings in this kindred indicate that severe familial hypertriglyceridemia manifested in childhood cannot be unequivocally equated with familial type I hyperlipoproteinemia (familial lipoprotein lipase deficiency).

5) Objective:

Characterization of the plasma lipoproteins and apolipoproteins in type III hyperlipoproteinemia.

Methods Employed:

Subjects with type III hyperlipoproteinemia were studied while consuming

ad libitum and high carbohydrate diets, and after short and long periods of caloric restriction. Their plasma lipoproteins were isolated, their chemical compositions determined, and their apolipoproteins fractionated by techniques described in previous annual reports. To date, only the very low density lipoproteins have been subjected to extensive analysis.

Major Findings:

a) Chemical composition of VLDL. Seven subjects with type III hyperlipoproteinemia were studied on one or more occasions when they were consuming ad libitum diets and taking no hypolipidemic medications. Their plasma cholesterols ranged from 184 to 586 mg/dl and triglycerides from 188 to 1500 mg/dl. The ratios of their plasma VLDL cholesterol concentrations to plasma triglycerides ranged from 0.29 to 0.59, fulfilling the current operational criteria for the diagnosis of type III hyperlipoproteinemia. Their average VLDL composition, expressed as percent dry weight of lipoprotein (range) was: free cholesterol - 6% (5-7%); cholesteryl esters - 28% (21-39%); triglyceride- 44% (32-55%); phospholipid - 17% (13-22%); and protein - 6% (5-8%). In four of the seven patients the cholesteryl ester content of the VLDL was within one s.d. of the VLDL cholesteryl ester content of six normolipidemic controls. However, the VLDL cholesteryl ester content in every case was higher than expected for the degree of hypertriglyceridemia, secondary undoubtedly to the presence of considerable "β-VLDL" in the whole type III VLDL fraction. As expected, the triglyceride content of the type III VLDL did not correlate well with the total plasma triglyceride concentration, a correlation that is good in normals and other forms of hypertriglyceridemia. One subject with a plasma triglyceride concentration of 318 mg/dl had a VLDL triglyceride content of 52%; the VLDL of another patient with a plasma triglyceride level of 1500 mg/dl contained only 44% triglyceride. Thus, while cholesterol enrichment of the VLDL is a common feature in type III hyperlipoproteinemia, the degree of enrichment varies considerably from patient to patient.

Four subjects were maintained for 10 days on diets high in carbohydrate (> 7 mg/kg/day) containing negligible quantities of cholesterol and fat. The cholesteryl ester content of their VLDL dropped 5-6% in three subjects and 10% in the fourth. This was accompanied by 2-9% rises in the VLDL triglyceride, but in every case the cholesteryl ester content of the VLDL was proportionately much greater than expected from the plasma triglyceride level. These results suggest that dietary cholesterol and fat are not pathogenetically implicated in the cholesteryl ester enrichment of VLDL in this disorder.

Three subjects were also maintained on severe caloric restriction for 7 to 30 days in an attempt to drastically inhibit their hepatic VLDL production. Plasma triglyceride levels in these patients fell to 114, 115 and 292 mg/dl by the end of the period of partial starvation. The cholesteryl ester content of the VLDL in these subjects fell to within normal limits and in such "treated" patients the "chemical abnormalities" characterizing type III hyperlipoproteinemia were no longer apparent. Additional studies under identical conditions are warranted, however, before these results can be generalized.

b) VLDL apolipoprotein composition. As noted in previous annual reports, the C-apolipoprotein content of type III VLDL usually, but not invariably, is relatively deficiency. Surprisingly, the total C-protein content does not appear directly related to the VLDL triglyceride content. One subject, for example, on three different dietary regimens had VLDL triglyceride contents of 43, 52 and 56%; the C-proteins accounted respectively for 33, 32 and 28% of the total protein.

The B protein content of type III VLDL estimated as that contained in the void volume on gel chromatography of the total protein, was invariably high. In normals and type V hyperlipoproteinemia we have found the VLDL-B protein content to range from approximately 25-35%. In 17 preparations of type III VLDL from 6 patients on a variety of dietary regimens, the VLDL-B protein content ranged from 39-59%.

Column chromatography has not permitted completely reliable estimations of the content of the so-called arginine-rich protein in type III VLDL. There appears little doubt, however, that it does not regularly account for 20% or more of the type III VLDL protein as reported by others. Our difficulty in obtaining accurate estimates has resulted from the elution of the protein in more than one column fraction and the obvious contamination of these fractions with other proteins of 20-40,000 M.W.

c) Composition of LDL and HDL. The lipid and protein composition of the following density classes have also been evaluated: LDL-1 ($1.006 < d < 1.035$), LDL-2 ($1.035 < d < 1.063$) and HDL ($1.063 < d < 1.21$ and $1.080 < d < 1.21$). Striking compositional varieties, not only between patients but in single patients on different dietary regimens, were documented. With the exception of one patient, the triglyceride content of the LDL-2 fraction was distinctly elevated to between 11-33% of lipoprotein dry weight. The triglyceride content of the HDL was also elevated (maximum of 17%) on ad libitum and high carbohydrate diets, but fell to within the normal range in all subjects who were treated with caloric restriction.

Significance to Biomedical Research and the Program of the Institute: Derangements of plasma lipoprotein metabolism, subtle or overt, familial or acquired, appear crucial in the initiation and perpetuation of the atherosclerotic lesion. The elucidation of pathogenetic mechanisms will depend heavily on both our understanding of lipoprotein structure and the factors controlling the plasma concentrations of these lipid transporting micelles. Studies in lipoprotein deficiency states have contributed immeasurably to the definition of the interrelationships of the various lipoprotein classes in normal lipid metabolism. The development of radioimmunoassay techniques for quantitating the apolipoproteins should greatly enhance our ability to refine the biochemical profiles of patients with coronary heart disease and identify the asymptomttic subjects who are at the greatest risk.

Proposed Course:

1) The radioimmunoassay for apoLP-A-I will be employed extensively in

studies of the total plasma concentration and lipoprotein distribution of this apolipoprotein in normals, subjects with hyperlipoproteinemia and hypolipoproteinemia, and individuals with angiographically proven coronary heart disease.

2) Techniques for the radioimmunoassay of the other apolipoproteins will be developed and applied to the same populations.

3) Analysis of the primary structure of apoLP-C-II will continue with an increased emphasis on its structure-function relationships.

4) Studies of the metabolism of infused normal high density lipoproteins and their effect on the now well-characterized lipoprotein and apolipoprotein abnormalities in Tangier disease will be conducted. Current efforts to further characterize the A-I and A-II apolipoproteins in this disorder will be continued.

5) Characterization of the various post-heparin lipolytic enzymes will be pursued with the aims of better definition of their substrate specificity, cofactor requirements and role in normal and abnormal lipoprotein metabolism.

6) The lipoproteins and apolipoproteins in type III hyperlipoproteinemia will continue to be the subject of intense investigation with particular emphasis on further characterization of the apolipoproteins of 20-40,000 M.W. which accumulate in the cholesterol-rich, triglyceride-poor lipoproteins of density < 1.019 g/ml.

7) Studies of the structure and metabolism of the lipoproteins and apolipoproteins of the rat, which were inactive in the last year, will be resumed.

Publications:

1. Yee, R. D., Herbert, P. N., Bergsma, D. R., and Biemer, J. J.: Atypical retinitis pigmentosa in familial hypobetalipoproteinemia. Amer. J. Ophthalmol., in press.

2. Witters, L. A., Herbert, P. N., Shulman, R. S., Krauss, R. M., and Levy, R. I.: Therapeutic failure in familial type II hyperlipoproteinemia. Metab., in press.

3. Herbert, P. N.: Hyperlipoproteinemia - a return to the basics. Hahnemann Endocrine Symposium, 1975, in press.

4. Herbert, P. N., and Fredrickson, D. S.: The hypobetalipoproteinemias. Handbuch der Inneren Medicine, in press.

This annual report is a combination of three previous reports: Z01 HL 02001-15, Z01 HL 02007-08 and Z01 HL 02008-06.

Annual Report of the
Molecular Hematology Branch
National Heart and Lung Institute
July 1, 1975 - June 30, 1976

The Molecular Hematology Branch has as its goal the treatment of human genetic diseases, specifically, thalassemia and sickle cell anemia. Several approaches have been initiated: first, complete fractionation of the erythroblast, which should allow a study of the factors involved in messenger RNA and protein synthesis. With this knowledge ways will be sought for influencing globin messenger RNA production in thalassemic cells. Second, the mechanism of globin gene switching is under active investigation using the animal model system of the hemoglobin A to hemoglobin C gene switch in sheep and goats. The goal is to learn the mechanism of this switch in order to understand how to obtain adult to fetal gene switching in patients who have normal fetal but abnormal adult hemoglobin. Third, somatic cell hybrids generated by fusion of erythroid cells and established cell lines have been used in an attempt to establish replicating cells capable of globin synthesis. These hybrids are being utilized to study regulation of expression of the globin genes. In addition, human-mouse hybrids containing one or more human chromosomes are being studied in order to determine the localization of the human alpha and beta globin structural genes. The Clinical Service of the Molecular Hematology Branch diagnoses, treats and studies patients with various blood cell disorders including thalassemia, sickle cell anemia and other hemolytic anemias. Several clinical protocols are under investigation. A summary of the results from each laboratory section follows:

SECTION ON CLINICAL HEMATOLOGY

The research efforts of this Section are organized to permit clinical studies of various disease states and their treatment and parallel investigation of the basic mechanism of red cell production, hemoglobin function, and molecular regulation of the globin genes in the laboratory. The consequences of iron overload on endocrine and cardiac function and the influence of chelation therapy is evaluated in chronically transfused patients, particularly those with thalassemia. At the same time the molecular defect in thalassemia is being elucidated as part of a general laboratory effort to elucidate the regulation of expression of the globin genes. Patients with thalassemia provide reticulocytes and bone marrow cells which are essential to these molecular studies. Similarly, the clinical characteristics of sickle cell disease, particularly in relationship to blood oxygen affinity, are being characterized in a large group of patients and the effect of altering blood oxygen affinity is being assessed. Parallel studies in the laboratory are permitting definition of the role of sickle hemoglobin gelation in determining blood oxygen affinity. Refinements of the technology for measuring blood oxygen affinity is being accomplished in order to permit quantitative determination of the influence of all factors on this important physiological parameter. A brief summary of the progress in this Section during the past year is as follows:

1. Clinical treatment of iron overload: The echocardiogram has been shown to be a very useful tool in detecting evidence of and following the progress of cardiac iron deposition. Chronic subcutaneous desferrioxamine administration has resulted in the excretion of large amounts of iron in heavily loaded patients.

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leading to the prediction that up to 5-10 gm of iron can be removed per year despite the need for continued transfusion therapy.

2. Molecular defect in thalassemia: The cell-free system for transcription of chromatin has been refined so that it should be possible to measure the relative synthesis of alpha and beta globin messenger RNA. This will permit determination as to whether the defect of beta thalassemia is at the transcriptional or post-transcriptional level in the nucleus.

3. Regulation of the globin genes: The genetically linked gamma (fetal hemoglobin) and beta (adult hemoglobin) genes in man and sheep serve as a model to investigate the mechanism of regulation of unique genes in eukaryotic cells. Current efforts are focused on the study of gene transcription. Preliminary evidence suggests that the human genes are transcriptionally regulated and this serves as an impetus to search for specific protein factors in chromatin which differentially influence the transcription of these closely related genes.

4. Hemoglobin switching in sheep and goats: The erythropoietin-induced switch from adult hemoglobin to hemoglobin C in these animals serves as a model to investigate the cellular basis of globin gene regulation. Our studies suggest that the immediate precursor to the committed erythroid cell may be the stage in erythropoiesis at which expression of individual globin genes is determined. Extension of the methodology for analysis of hemoglobin switching in these animals to the analysis of the fetal to adult hemoglobin switch in man is anticipated during the next several months.

The general goal of the last three projects is to provide specific insights into the mechanism of regulation of the globin genes with the desire to influence the relative synthesis of various hemoglobins in man. Thus, treatment of both beta thalassemia and the several hemoglobinopathies, of which sickle cell disease is a prototype, could be successfully accomplished if increased fetal hemoglobin production could be obtained. Since there are cells in the bone marrow of humans committed to production of Hb F, this therapeutic goal could be accomplished by finding means to enhance the relative number of blood cells produced by these precursors. Alternately, insight into the molecular mechanism of globin gene regulation might lead to the development of specific substances which could influence this mechanism in vivo.

5. Cardiac Hemolytic Anemia: Efforts to improve the medical management of this complication of cardiac valve replacement continue. Evidence has been obtained which implicates that relative bone marrow failure may be an important factor in producing anemia in some patients with hemolysis.

6. Alteration of blood oxygen affinity in the treatment of sickle cell anemia: Many patients with sickle cell disease have lowered blood oxygen affinity allowing them to release oxygen more readily to their tissues. Our studies have shown that this phenomenon is at least partially related to intracellular hemoglobin gelation itself. A survey of approximately 20 patients suggests that those who are most "anemic" have the lowest blood oxygen affinity. Thus these patients may in fact be able to deliver normal amounts of oxygen to their tissues despite a lowered hemoglobin concentration in the blood. Since any agent which will influence hemoglobin gelation is likely to simultaneously increase blood oxygen affinity, it seems imperative to evaluate the effect of this alteration clinically.

7. Regulation of the respiratory function of blood: Highly sophisticated methodology has been evolved which permits precise measurement of the complete blood oxygen affinity curve. Cooperativity of the hemoglobin tetramer and the influence of various allosteric effectors, e.g., e,2-diphosphoglycerate, pH, and pCO_2 can be determined precisely at various positions in the curve. This methodology will be applied to the study of blood oxygen affinity in animals and also in man as outlined above.

Precise understanding of the regulation of blood oxygen affinity is likely to have application beyond the immediate scope of hematology. Thus, oxygen exchange in the lungs, particularly in those patients with compromised pulmonary function, is likely to be influenced by blood oxygen affinity. Similarly, delivery of oxygen to tissues in patients with compromised cardiac function or circulatory status will similarly be influenced by this important variable.

SECTION ON MOLECULAR GENETICS

The Section on Molecular Genetics is studying the molecular basis of gene expression. Two approaches are being used: first, examination of the regulation of globin gene expression in somatic cell hybrids by a combination of cell biology and molecular biology techniques; second, the synthesis of full-length globin genes for eventual insertion into mammalian cells.

Somatic cell hybrids are formed by virus-induced fusion of human (or animal) erythroid cells with mouse erythroleukemia cells. These hybrids lose certain chromosomes during cell division. The presence or absence of the globin genes is correlated with the chromosome composition of the hybrid cells. In this way the chromosomal assignment of the globin loci should be determined. Furthermore, by measuring the expression of the globin genes (i.e., by quantitating globin messenger RNA), factors affecting globin gene expression in intact cells can be studied.

The section has succeeded, during the past year, in obtaining reaction conditions such that multiple copies of complete globin gene DNA can be synthesized by each enzyme molecule of reverse transcriptase. This finding greatly increases the yield of material and opens the way for a major effort towards synthesizing complete double-stranded globin gene molecules for potential insertion into mammalian cells.

UNIT ON PROTEIN BIOSYNTHESIS

The goals of this unit are: 1) to identify and purify the protein and nucleic acid components functional in protein biosynthesis; 2) to establish the precise function of each component; 3) to evaluate possibilities for regulating the synthesis of alpha and beta globin chains both *in vitro* and *in vivo*. The first phase of this program is nearing completion. Most if not all the activities required for the initiation of protein biosynthesis have been identified and the proteins corresponding to six of the initiation factors (IF) have been purified to homogeneity. The only two elongation factors (EF) known to be required for polypeptide elongation (EF-1 and EF-2) have also been prepared in homogeneous form.

To date, most of the experimentation to evaluate the function of the components required for protein biosynthesis has been with model templates, either

AUG codon or poly(U). These studies implicate IF-M1 and IF-MP in the directing of initiator tRNA (Met-$tRNA_f$) binding to the 40S ribosomal subunit. The subsequent joining of 40S and 60S ribosomal subunits requires IF-M2A, IF-M2Bα and IF-M2Bβ. Work has begun on evaluating the role of the above proteins plus IF-M3, IF-M4, and IF-M5 on the translation of natural globin mRNA.

Due to the scarcity of purified components, studies on possible regulation at the translational level have been limited. Recent experiments in our laboratory (and also several non-NIH collaborators) indicate that IF-MP and IF-M3 may participate in translational regulation. IF-MP is capable of reactivating hemin deficient lysate systems which normally fail to synthesize globin chains in the absence of added hemin. IF-M3 appears to bind selectively globin mRNA (perhaps specifically at the 5' end). Work is also in progress to evaluate the role of reticulocyte protein kinases on the activation or inactivation of globin chain synthesis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL02211-02 MHB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Alteration of Blood Oxygen Affinity in the Treatment of Sickle Cell Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI.:	R. M. Winslow	Senior Investigator	MHB NHLI
OTHER:	R. E. Jackson	Special Expert	MHB NHLI
	M. L. Swenberg	Research Chemist	MHB NHLI
	A. W. Nienhuis	Chief, Sect. on Clin. Hemat.	MHB NHLI
	J. Fulmer	Chief, Clinical Service	PB NHLI
	S. Charache	Visiting Scientist, Johns Hopkins	University Baltimore, Md.

COOPERATING UNITS (if any)
Pulmonary Branch, NHLI; Laboratory of Technical Development, NHLI; and Laboratory of Molecular

LAB/BRANCH
Molecular Hematology Branch

SECTION
Section on Clinical Hematology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	none

SUMMARY OF WORK (200 words or less - underline keywords)

Many current lines of research suggest that the increase of blood oxygen affinity in this disease might diminish sickling; however, it is also possible that the increase of blood oxygen affinity in patients could lead to a worsening of their clinical state due to decreased oxygen affinity. The objective of this work is to evaluate the effect of alteration of blood oxygen affinity in patients with sickle cell anemia.

PHS-6040
(12-75)

Methods: An attempt is made to evaluate the clinical status of a group of patients representing a spectrum of severity. Routine testing is done to assess the state of damage in various organ systems. We are attempting to develop a quantitative codification of this information so it can be assessed by the computer to draw correlations between our clinical and laboratory data.

1. Exercise testing has been carried out in three homozygous S patients.

2. Blood oxygen affinity is measured according to the newly developed and further defined method of the addition of hydrogen peroxide to whole blood containing small amounts of catalase. This method has been developed in conjunction with the Laboratory of Technical Development, NHLI.

Findings:

1. The relationship between the number of irreversiby sickled cells, degree of anemia and blood p50 has been extended to approximately 20 patients. This relationship does not now appear to be as close as was previously throught. The cause and effect relationship for these two parameters is not established.

2. Exercise tolerance, based upon available exercise testing, is very difficult to assess. New methods for the measurement of anerobic threshold will probably be required in order to quantitate exercise tolerance.

3. The position and shape of the whole blood oxygen dissociation curve in sickle cell anemia cannot be explained on the basis of known effects of pH and 2,3-DPG: the shape of the curve is unique. The most significant result of our study to date, therefore, is that the regulation of blood oxygen affinity in sickle cell anemia is probably the result of the intracellular hemoglobin S aggregation itself and that there is a tight linkage between hemoglobin S gelation and oxygen affinity. Therefore, our measurement of blood oxygen affinity in patients with sickle cell anemia probably reflects actual in vivo aggregation of hemoglobin S and therefore, sickling.

4. Carbon monoxide has not yet been administered to patients under the original protocol. Instead, we have given oral sodium bicarbonate to several patients in an effort to increase their blood oxygen affinity. It was initially thought that the mechanism of action of this agent would be by increasing blood pH and therefore increasing the blood oxygen affinity by the well known Bohr effect. However, preliminary experiments suggest that the change in blood p50 is far greater that would be expected on the basis of the very small changes in blood pH which we have observed. We have further found that the method of administration of sodium bicarbonate is very important and that its duration of action is exceedingly short, probably on the order of a few hours. When given intravenously there is no effect whatever on blood oxygen affinity, and unless it is given very quickly as a pulse infusion.

Significance to Biomedical Research and the Program of the Institute: Sickle cell anemia is a disease of great current research interest. A large effort is being made to identify agents which will interfere with or inhibit hemoglobin S aggregation. Our studies indicate that partial reduction of hemoglobin S aggregation will have the consequence of increasing blood oxygen affinity,

which, in turn, will lead to diminished oxygen delivery unless compensatory increase in the red cell mass occurs. This, of itself, could have deleterious effects because of increased blood viscosity. Therefore, we feel that it is exceedingly important to carefully define the clinical consequences of the alteration of blood oxygen affinity in sickle cell anemia before further clinical trials of such drugs is undertaken.

Proposed Course of the Project:

1. We will continue to evaluate the effectors of whole blood oxygen affinity in sickle cell anemia by application of the method of the measurement of the whole blood oxygen dissociation curve and correlation with the measurement of dissociation curves of purified hemoglobin solutions and hemolysates. We will attempt to answer the question of whether the shape and position of whole blood oxygen dissociation curve of sickle cell anemia can be accounted for by the sum of the effects of the various cellular components on the oxygen affinity of pure hemoglobin S solutions.

2. We will continue the study of the effect of sodium bicarbonate on the sickling phenomenon, oxygen affinity of whole blood, and possibly at a later date, on the clinical course of sickle cell anemia.

3. We will attempt to develop, with the Pulmonary Branch, methods for measuring the anerobic threshold of patients during exercise so that we can quantitate more precisely the effect of manipulation of blood oxygen dissociation curves on peripheral oxygen delivery.

4. New methods are being developed in conjunction with the Laboratory of Technical Development for the measurement of kinetic properties of sickle cell blood and purified hemoglobins.

5. We will continue the accumulation of clinical information in an attempt to facilitate correlation between physiologic and clinical measurement.

Publications:

1. Winslow, R.M.: Blood oxygen equilibrium studies in sickle cell anemia. Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, Dallas, Texas, 1975. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02201-04 MHB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanism of Hemoglobin Biosynthesis in Cell-Free Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.C. Merrick	Research Chemist	MHB NHLI
OTHER:	B. Safer	Staff Associate	MHB NHLI
	S. Adams	Chemist	MHB NHLI
	W. Kemper	Chemist	MHB NHLI
	M. Lloyd	Chemist	MHB NHLI
	K. Berry	Biologist	MHB NHLI
	J. Osborne	Staff Fellow	MDB NHLI
	D. Shafritz	Assoc. Prof.	Albert Einstein
	J. Traugh	Assoc. Prof.	Univ. California

COOPERATING UNITS (if any)

Albert Einstein College of Medicine
Department of Biochemistry, University of California (Riverside)
Molecular Disease Branch

LAB/BRANCH

Molecular Hematology Branch

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIH, NHLI, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	5.0	2.0

SUMMARY OF WORK (200 words or less - underline keywords)

All the protein components required for the formation of an initiation complex utilizing artificial templates (AUG triplet or Poly (U)) have been purified to homogeneity (IF-M1, IF-MP, IF-M2A, IF-M2Bα, IF-M2Bβ, EF-1, EF-2). There appear to be two distinct pathways for initiation complex formation, one utilizing IF-MP to direct the binding of initiator tRNA, the other using IF-M1. Despite differences between the requirements for IF-MP or IF-M1 dependent binding of initiator tRNA to 40S subunits, the subsequent requirements for GTP, AUG, IF-M2A, IF-M2Bα, IF-M2Bβ, and 60S subunits to achieve an 80S initiation complex are identical.

More recent experiments have indicated that three additional initiation factors (M3, M4, M5) are required for the translation of globin mRNA. At this point it is not clear how each of the factors functions. There is evidence to suggest that IF-M3 directly binds mRNA, specifically at the 5' end of the mRNA molecule. Neither IF-M4 nor IF-M5 appears capable of binding globin mRNA. Whether these latter factors function in the assembly or regulation of an 80S initiation complex containing globin mRNA is currently under investigation.

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PHS-6040
(12-75)

Objectives: The long range goal of this study is to define the regulation and biosynthesis of α and β globin chains. The initial phase of this study is to define the components required for the utilization of artificial mRNA templates. The second phase is to define the components required for the translation of globin mRNA. The third phase is the study of the regulation of globin chain synthesis. Each phase requires identification and purification of each of the protein components as well as the preparation and utilization of both unlabeled and labeled nucleic acid components.

Methods: Purification of the separate protein components has been achieved using standard techniques such as ion-exchange column chromatography, gel filtration, salt fractionation, preparative gel electrophoresis, and sucrose density gradient centrifugation. RNA components were purified by gel filtration, reversed phase column chromatography and/or oligo (dT) cellulose chromatography. The physical properties of initiation or elongation factors (IF and EF, respectively) were studied using gel electrophoresis, gel filtration, analytical ultracentrifugation and amino acid analysis. Biological characterization of each component was performed using radioactive amino acids and/or nucleic acids followed by analysis of products using Millipore filters, sucrose gradients, or gel electrophoresis.

Major Findings: (1) IF-M2Bα and IF-M2β have been purified to homogeneity. The molecular weight of both proteins has been determined by gel filtration, SDS gel electrophoresis and analytical ultracentrifugation to be about 15,000 and 17,000 (IF-M2Bα and IF-M2Bβ, respectively). Amino acid analysis indicated a few unusual features: a) IF-M2Bα contains no tryptophan; b) IF-M2Bβ contains almost 20% glutamic acid (as glutamic acid plus glutamine); c) IF-M2Bα has the highest content of sulphur amino acids of all the initiation factors. In addition, IF-M2Bα is capable of self-association. Both factors seem to be required for all initiation reactions which require joining of the 40S and 60S subunits, however, it has not been possible to demonstrate an IF-M2Bα requirement in globin chain synthesis because of the high level of IF-M2Bα present in the crude amino acyl-tRNA synthetase preparation.

(2) IF-MP was previously reported to contain two subunits, 35,000 and 55,000 daltons. Analysis by acid urea gel electrophoresis and a different SDS gel system (that of Laemmli instead of the Weber and Osborne procedure) indicate that IF-MP consists of three polypeptide chains. Studies are in progress to determine whether IF-MP exists as a trimer ($\alpha\alpha'\beta$) or a tetramer ($\alpha\alpha'\beta_2$).

(3) Two additional initiation factors have been found and highly purified. IF-M4 is a single polypeptide of molecular weight 48,000. IF-M5 is a high molecular weight complex (>500,000) which consists of at least 6-7 polypeptide chains. Both factors are required for the translation of globin mRNA although neither factor appears to bind globin mRNA directly.

(4) A study of the ability of various initiation factors to bind natural mRNAs has shown that IF-MP, IF-M2A, and IF-M3 can bind VSV, histone, or EMC mRNA. However, only the binding by IF-M3 was inhibited by 7 methyl GMP, an analog of the capped 5' end of natural mRNAs. As both IF-MP and IF-M2A are bound tightly to phosphocellulose, it is not possible at this point to determine the significance of the mRNA binding observed with these two factors.

(5) Reticulocyte EF-1 has been prepared in apparent homogeneous form. The enzyme is a high molecular weight (about 450,000) aggregate and is composed of 53,000, 50,000, and 30,000 dalton polypeptides. The enzyme contains little or no phospholipid, unlike other EF-1s prepared from different tissues. The protein can be disaggregated into an active low molecular weight form by treatment of the protein with either partially purified phospholipase C or elastase.

Significance to Biomedical Research and Institute Program

An understanding of the regulation of gene expression is a key to understanding normal and abnormal cell function. Recent studies in the field of protein biosynthesis have indicated that regulation at the translational level does occur. The most clear cut example is the hemin control of α and β globin chains. The ability to localize the point of control may permit a control of the synthesis of limiting or excess globin chains.

Proposed Course of Project: Future studies will concentrate on the translation of globin mRNA. Purification of the protein factors specifically required for globin mRNA translation will be continued until IF-M3, IF-M4, and IF-M5 have been prepared in homogeneous form. The role of proteins which are part of mRNP complexes will also be investigated. With purified initiation factors, mRNAs, initiator tRNA, and ribosomal subunits (40S and 60S), studies will be aimed at determining the step by step formation of an initiation complex with globin mRNA.

Publications:

1. Clemens, M.J., Safer, B., Merrick, W.C., Anderson, W.F., and London, I.M.: Inhibition of protein synthesis in rabbit reticulocytes by double-stranded RNA and oxidized glutathione: Evidence for an indirect mode of action on polypeptide chain initiation. Proc. Nat. Acad. Sci. USA 72, 1286-1290, 1975.

2. Merrick, W.C., Kemper, W.M., and Anderson, W.F.: Purification and characterization of homogeneous initiation factor M2A form rabbit reticulocytes. J. Biol. Chem. 250, 5556-5562, 1975.

3. Safer, B., Anderson, W.F., and Merrick, W.C.: Purification and physical properties of homogeneous initiation factor MP from rabbit reticulocytes. J. Biol. Chem. 250, 9067-9075, 1975.

4. Safer, B., Adams, S.L., Anderson, W.F., and Merrick, W.C.: Binding of Met-tRNA$_f$ and GTP to homogeneous initiation factor MP. J. Biol. Chem. 250, 9076-9082, 1975.

5. Adams, S.L., Safer, B., Anderson, W.F., and Merrick, W.C.: Eukaryotic initiation complex formation: Evidence for two distinct pathways. J. Biol. Chem. 250, 9083-9089, 1975.

6. Kemper, W.M., Merrick, W.C., Redfield, B., Liu, C-K., Weissbach, H.: Purification and properties of rabbit reticulocyte elongation factor 1. Arch. Biochem. Biophys. (in press).

7. Shafritz, D.A., Weinstein, J.A., Safer, B., Merrick, W.C., Weber, L.A., Hickey, E.D., and Baglioni, C.: Recognition of Eukaryotic messenger RNA by initiation factor IF-M3: evidence for a role of 7-methyl guanosine-5' phosphate group. Nature (in press).

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8. Safer, B., Adams, S.L., Kemper, W.M., Berry, K.W., Lloyd, M., and Merrick, W.C.: Purification and characterization of two initiation factors required for maximal activity of a highly fractionated globin mRNA translation system. Proc. Nat. Acad. Sci. USA (in press).

9. Kemper, W.M., Berry, K.W., and Merrick, W.C.: Purification and properties of rabbit reticulocyte protein synthesis initiation factors M2Bα and M2Bβ. J. Biol. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02202-04 MHB

PERIOD COVERED

July 1, 1975 to June 30, 1976.

TITLE OF PROJECT (80 characters or less)

Evolution of the Protein Synthesizing Machinery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.C. Merrick	Research Chemist	MHB NHLI
OTHER:	B. Safer	Staff Associate	MHB NHLI
	S. Adams	Chemist	MHB NHLI
	W.F. Anderson	Chief, MHB	MHB NHLI
	S. Ochoa	Distinguished Member	Roche Inst. Mol. Biol.
	C. Nombela	Research Fellow	Roche Inst. Mol. Biol.
	W. Filipowicz	Postdoctoral Fellow	Roche Inst. Mol. Biol.

COOPERATING UNITS (if any)

Roche Institute of Molecular Biology

LAB/BRANCH

Molecular Hematology Branch

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIH, NHLI, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

From studies using highly purified or homogeneous preparations of rabbit reticulocyte initiation factors, it appears that the mechanism of initiation complex formation is the same in both A. salina (brine shrimp) and rabbit reticulocytes. Further, the high degree of replacement of A. salina components by the more purified reticulocyte initiation factors (in an assay system using A. salina ribosomes) indicates that despite the wide evolutionary gulf between A. salina and rabbits, there appears to have been a restricted divergence of the more than 15 proteins (polypeptide chains) which are required for initiation complex formation.

890

PHS-6040
(12-75)

Objectives: The purpose of this study is to evaluate the possible differences between various eukaryotic and prokaryotic organisms with respect to the initiation of protein synthesis. Both mechanism and components are being tested for similarities.

Methods: Components from both prokaryotic and eukaryotic organisms are tested for their ability to form initiation complexes using assay techniques developed or modified from previous reports.

Major Findings:

1. The initiation factor requirements for the translation of globin mRNA in a A. salina protein synthesizing system appear to be quite similar to those of the rabbit reticulocyte system. In particular, reticulocyte IF-MP, IF-M3, IF-M2A, and IF-M2B have been shown to be functional in the A. salina system.

2. Antibody to A. salina EIF-1 which inactivates EIF-1 directed binding of aminoacyl-tRNA to 40S subunits does not inhibit the translation of globin mRNA in the A. salina assay system. This observation suggests that EIF-1 (and it's reticulocyte counterpart, IF-M1) is not required for the translation of natural mRNAs.

3. Using the artificial initiating codon AUG, it was demonstrated that A. salina (like reticulocytes) possess two pathways for the formation of a puromycin sensitive 80S initiation complex. Unlike the reticulocyte system, the initiation factor requirements are quite different. While both IF-MP and IF-M1 can direct the binding of initiator tRNA to 40S subunits, only for IF-MP is there a requirement for additional initiation factors for the formation of an 80S initiation complex.

Significance to Biomedical Research and Institute Program:

The ability of initiation factors from different sources to interact details both differences and similarities between these protein synthesizing systems. The potential capability to distinguish between different prokaryotic and eukaryotic pathways for the formation of an 80S initiation complex may form the basis for the formulation of new treatments for disease stemming from bacterial or viral infections or parasitic infestations.

Proposed Course of Project:

With more highly purified components from A. salina, experiments testing exact degree of cross-reactivity will continue to be performed. In addition, wheat germ and possibly E. coli protein synthesizing systems will be used along with a reticulocyte system to evaluate the activity of different prokaryotic and eukaryotic mRNAs in these translation systems.

Publications:

1. Filipowicz, W., Sierra, J.M., Nombela, C., Ochoa, S., Merrick, W.C., and Anderson, W.F.: Polypeptide chain initiation in eukaryotes: Initiation factor requirements for translation of natural messengers. Proc. Nat. Acad. Sci. USA 73, 44-48, 1976.

2. Nombela, C., Nombela, N.A., Ochoa, S., Safer, B., Anderson, W.F., and Merrick, W.C.: Polypeptide chain initiation in eukaryotes: Mechanism of formation of initiation complex. Proc. Nat. Acad. Sci. USA 73, 298-301, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL02203-04 MHB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Molecular Defect in Thalassemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. W. Nienhuis, Chief, Section on Clinical Hematology, MHB, NHLI
Other: A. W. Steggles, Visiting Associate MHB, NHLI
E. J. Benz, Research Associate MHB, NHLI
W. F. Anderson, Chief MHB, NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Molecular Hematology Branch

SECTION
Section on Clinical Hematology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to understand the molecular lesions which result in a deficiency in globin synthesis in the various thalassemia syndromes. Current effort is concentrated on studying transcription of the individual globin genes in chromatin and the processing of RNA in the nucleus. Chromatin from patients with beta thalassmia is transcribed in vitro and the relative synthesis of alpha and beta globin mRNA sequences is determined. Nuclear RNA is isolated and the relative sequence ratio for alpha and beta globin mRNA is determined in various size fractions and compared to that found in the cytoplasm.

892

PHS-6040
(12-75)

Objective: The thalassemias are disease states characterized by a reduced synthesis of either the alpha or beta globin polypeptides of hemoglobin resulting in a deficiency of hemoglobin in the red cells and therefore a hypochromic microcytic anemia. In addition, there is an excess of the globin which is produced normally, either alpha chains in beta thalassemia, or beta chains in alpha thalassemia. This excess globin precipitates within the erythroid cells leading both to ineffective erythropoiesis and accelerated destruction of red cells in the peripheral blood. In many of the thalassemia syndromes there is reduced or absent mRNA for the globin which is produced in deficient amounts. Thus the molecular defect is likely to be in transcription of the gene within the nucleus or processing of the immediate product of transcription prior to its emergence in the cytoplasm as globin mRNA. The normal mechanism for regulation of human globin mRNA production and the nature of the defect in thalassemia cells are being investigated.

Methods: Globin mRNA is purified from patients with hemolytic anemia, beta thalassemia, and alpha thalassemia (Hb H disease). Globin complementary DNAs are synthesized using the viral enzyme RNA-directed DNA polymerase. These probes are used in hybridization reactions to quantitate globin mRNA sequences. A cDNA specific for the globin unique to Hb F (gamma globin) is prepared by cycling cDNA made with newborn reticulocyte mRNA over hydroxylapatite after hybridization to adult reticulocyte mRNA. In some instances, adult reticulocyte mRNA is fractionated to yield alpha and beta globin mRNA by polyacrylamide gel electrophoresis in formamide and specific alpha and beta cDNAs are synthesized. DNA, chromatin, and nuclear RNA are isolated from cells of patients with hemolytic anemia or thalassemia.

Major Findings:

1. DNA was purified from the bone marrow cells of a patient with beta thalassemia. The alpha and beta globin gene sequence content was found to be equal to that of normal DNA. Thus there is no evidence for gene deletion in DNA from the affected tissues of this patient.

2. Chromatin from a patient with beta thalassemia was transcribed in vitro. The initial results indicate that there is imbalance in the transcription of alpha and beta globin genes. This was true only when highly purified bacterial RNA polymerase containing the specificity subunit, sigma factor, was employed in the transcription reaction. This result would suggest that the lesion in this patient was at the transcriptional level.

3. Chromatin from cells of an adult with erythroid hyperplasia was transcribed in vitro. Adult (alpha and beta) sequences were produced, but no gamma globin sequences were transcribed. This suggests that the individual human globin genes may be regulated at the transcriptional level.

Significance to Biomedical Research and the Program of the Institute: The thalassemias are severe hereditary diseases which affect more individuals worldwide than any other single gene defect. The definition of the molecular lesion in these patients may lead to rational efforts to devise therapeutic intervention, particularly for those individuals with homozygous beta

thalassemia who require lifelong transfusion therapy.

Proposed Course of the Project: Chromatin from additional patients with beta thalassemia will be transcribed in vitro and the relative alpha and beta mRNA sequences determined. If imbalance in the relative amounts of alpha and beta globin mRNA sequences is confirmed, an effort will be made to determine whether this is due to abnormal initiation of beta globin mRNA synthesis or due to an alteration in the binding of chromosomal proteins.

Publications:

1. Velez, R., Kantor, J.A., Picciano, D.J., Anderson, W.F., and Nienhuis, A.W.: Alpha and beta complementary DNAs of human and rabbit: Specificity of hybridization. J. Biol. Chem. 250: 3193-3198, 1975.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02204-04 MHB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Hemoglobin Switching in Sheep and Goats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Barker, Senior Staff Fellow	MHB, NHLI
	R. Croissant, Post-Doctoral Fellow	MHB, NHLI
	A. W. Nienhuis, Chief, Section on Clinical Hematology	MHB, NHLI
Other:	E. J. Benz, Jr., Research Associate	MHB, NHLI
	J. E. Pierce, Chief, Laboratory on Animal Surgery	NHLI
	L. Stuart, Chief, Ungulate Section, NIH Animal Center	
	W. F. Anderson, Chief, Molecular Hematology Branch	NHLI

COOPERATING UNITS (if any)

Section on Laboratory Medicine and Surgery, NHLI and Ungulate Section, NIH Animal Center

LAB/BRANCH

Molecular Hematology Branch

SECTION

Section on Clinical Hematology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to define the cellular and molecular mechanism of the hemoglobin switch which occurs in sheep and goats in response to high concentrations of erythropoietin. Current efforts are focused on defining the precise stage in erythropoiesis at which the switch is regulated and specifically determining whether this occurs before or simultaneously with the onset of globin mRNA synthesis. The ontogeny of hemoglobin synthesis during fetal development is being defined. The effect of erythropoietin on synthesis of the individual globins is being studied in hepatic and bone marrow cells of fetuses at various times of gestation. The types of hemoglobin synthesized in individual erythroid colonies in vitro is under investigation in an effort to determine whether erythropoietin mediates the switch by intracellular modulation of expression of the individual globin genes or by selection among various precursor cells which have been predetermined as to which globin genes their progeny will express.

PHS-6040
(12-75)

Objectives: This project utilizes the erythropoietin-induced switch in hemoglobin phenotype which occurs in sheep and goats as a model system for understanding factors which regulate individual globin gene expression in erythroid cells. Both the cellular events accompanying differentiation and hemoglobin synthesis and the intracellular events related to the regulation of transcription or globin mRNA production are being explored. These studies will be applied to human bone marrow cells when pertinent techniques have been evolved.

Methods: Bone marrow is obtained from normal young animals or those previously injected with erythropoietin. In addition, erythroid cells are obtained from fetal liver, spleen, and bone marrow. The cells are grown in plasma clot culture. Bone marrow and fetal liver are fractionated by unit gravity sedimentation to obtain precursors at various stages of maturation. A population of early precursor cells is also obtained by differential immune lysis. Total globin synthesis in culture is quantitated by measurement of incorporation of [^{3}H]leucine and the type and amount of each globin chain is determined by chromatography on an ion exchange column. Techniques are being developed to recover individual erythroid colonies after culture and to quantitate synthesis of the individual globins in single colonies. Accumulation of cytoplasmic globin mRNA is studied in immature erythroid precursor cells obtained by cell fractionation. Globin mRNA sequences are quantitated by hybridization to globin complementary DNA.

Major Findings:

1. Substantial amounts of Hb C synthesis has been obtained in culture of sheep bone marrow but only after five days incubation in vitro. It appears that the initial cohort of colony-forming cells derived from bone marrow is committed as to its hemoglobin phenotype. Thus colonies active in hemoglobin production between two and four days in vitro synthesize predominantly adult globins. Between three and five days a cohort of very large colonies developed which appeared to synthesize a substantial amount of Hb C (beta^C). These are presumably derived from an earlier precursor than the smaller colonies appearing after two to four days in culture. In vivo injection of erythropoietin appears to increase the number of colony forming cells in the bone marrow over a 24 hr. period and these appear to acquire a commitment to Hb C production as revealed by the globins synthesized subsequently in vitro. This commitment is retained in vitro even if the incubation is carried out at low erythropoietin concentration.

2. Erythroid colonies have been derived from tissues of fetal sheep at several stages of gestation extending from 40-140 days (normal gestation period 140 days). The liver is the predominant erythropoietic organ until approximately 80-90 days. Adult globin synthesis appears to be well established by 130-140 days, concomitant with erythropoiesis becoming established in bone marrow. An effort to induce Hb C synthesis in cultures of fetal sheep from an earlier stage have met to date with partial success, e.g., a small amount of radioactivity co-migrates with an authentic beta^C globin marker on ion exchange chromatography.

3. A fraction of immature fetal liver cells has been obtained by immune lysis of more mature precursors or by unit gravity sedimentation. These early erythroid cells contain relatively little globin mRNA. Incubation of these cells with erythropoietin results in a marked increase in globin mRNA sequences.

Significance to Biomedical Research and to Institute Program: These studies are designed to determine the stage of erythropoiesis at which the globin genes are regulated. The mechanism of action of erythropoietin will be elucidated. The methodology and basic information derived from these studies will be applied to the switch from fetal hemoglobin synthesis to adult hemoglobin in man.

Proposed Course of the Project: The exact cellular stage at which the hemoglobin switch is regulated will be determined. An effort will be made to purify this population of cells to study the molecular mechanism by which commitment occurs. Synthesis of the individual hemoglobins in single colonies will be quantitated to determine whether erythropoietin produces a switch by selecting among various committed precursors or by an intracellular modulation mechanism within early cells. The effect of erythropoietin concentration on the globins synthesized in colonies from fetal tissue will be further defined. Using cDNA probes specific for gamma and beta globin (see Project No. Z01 HL 02206-03 MHB) mRNA, the type of mRNA sequences induced by erythropoietin in immature precursor cells will be determined.

Publications:

1. Nienhuis, A. W., Barker, J.E., and Anderson, W.F. The effects of erythropoietin on hemoglobin synthesis. In "Kidney Hormones, Volume 2" (J. Fisher, editor), in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL02206-03 MHB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of the Globin Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. W. Steggles, Visiting Associate
E. J. Benz, Research Associate
A. W. Nienhuis, Chief, Section on Clinical Hematology, MHB, NHLI

Other: J. Barker, Senior Staff Fellow, MHB, NHLI
D. M. Miller, Clinical Associate, MHB, NHLI
W. F. Anderson, Chief, Molecular Hematology Branch, NHLI
J. B. Pierce, Chief, Laboratory of Animal Surgery, NHLI
Leonard Stuart

COOPERATING UNITS (if any)

Laboratory of Animal Surgery, NHLI, NIH Ungulate Unit, NIH Animal Center

LAB/BRANCH
Molecular Hematology Branch

SECTION
Section on Clinical Hematology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to define the mechanism of regulation of the globin genes in erythroid cells. Transcriptional regulation is being studied specifically by isolation of chromatin from various erythroid tissues of sheep and rabbit. The chromatin is transcribed in vitro with RNA polymerase, and synthesis of mRNA sequences measured. Complementary DNA specific for the individual globin mRNA of sheep (beta for adult hemoglobin and gamma for fetal hemoglobin) are being prepared so that transcription of the individual genes may be studied. Techniques are also being developed to purify specific protein factors which may be important in transcriptional regulation.

898

PHS-6040
(12-75)

Objective: The objective of this project is to determine the normal mechanism of regulation of globin genes in erythroid cells. There are several different levels at which regulation might occur, e.g., gene transcription, nuclear RNA processing, or globin mRNA translation. The globin genes of sheep serve as a suitable model for investigating these phenomena since the several individual genes are expressed only under specific conditions. Thus the gamma globin gene (Hb F) is expressed during fetal life, the adult beta globin genes are expressed in adult animals while the beta^{C} globin gene (Hb C) is expressed in newborn animals and in adults made anemic. Extensive evidence exists which indicate that synthesis of these globins is not regulated at the translational level. Thus our current efforts are focused on study of transcription of these genes in chromatin in an effort to determine whether regulation of the individual globin genes occurs at this level.

Methods: Chromatin is purified from the liver of fetal sheep and the bone marrow of young sheep injected with phenylhydrazine. These tissues are composed primarily of erythroid cells. Bone marrow cells from rabbits injected with phenylhydrazine are also used in experiments designed to optimize chromatin preparation or the conditions for RNA transcription. Highly purified bacterial RNA polymerase is prepared from E. coli by a series of steps including DNA affinity chromatography. Mammalian polymerase is obtained from sheep liver. The quantity of globin mRNA sequences transcribed in vitro is determined by hybridization to globin complementary DNA prepared by using reticulocyte globin mRNA as a template for RNA-directed DNA polymerase. Chromatin proteins are dissociated from DNA in concentrated salt and urea and purified by filtration or ion exchange chromatography. Reconstitution of chromatin is accomplished by gradient dialysis.

Major Findings:

1. Globin gene transcription has been shown to be symmetrical in vitro when both bacterial and mammalian RNA polymerases are employed. Thus both the globin gene and the DNA strand complementary to it are transcribed despite the fact that no RNA sequences complementary to the anti-strand are detected in RNA synthesized in vivo. Purification of the mammalian RNA polymerase to approximately one-third homogeneity does not result in increased specificity of transcription.

2. Chromatin from sheep bone marrow and sheep fetal liver has been transcribed in vitro and globin mRNA sequences detected.

3. Preliminary evidence suggests that it will be possible to reconstitute erythroid chromosomal proteins with DNA from other tissues and obtain chromatin capable of supporting transcription of the globin genes.

4. The thermal stability of heterologous hybrids formed between adult sheep globin cDNA (α, β^{B}) and fetal mRNA (α, γ) has been compared to that of the homologous hybrid between adult mRNA and cDNA. The heterologous hybrid (γ mRNA to β cDNA) melts with a T_M 7° lower than that of the homologous hybrid. Thus the nucleotide sequence of the mRNAs for γ and β globin appears to differ to an extent no greater than that predicted from the number of amino acid

differences (29) between the two chains. Beta cDNA has been purified to remove alpha sequences by hybridization of adult cDNA (α, β^B) to fetal mRNA (α, γ), melting of the γ-β hybrids and fractionation of the single stranded (β) and double stranded cDNA (α) by hydroxylapatite chromatography. Preliminary experiments suggest that it will be possible to devise hybridization conditions sufficiently stringent so that the beta cDNA may be used specifically to quantitate beta mRNA sequences in the presence of those for gamma globin.

Similarly, it appears possible to prepare a probe specific for gamma mRNA sequences. These specific probes (β and γ) are essential for the projected studies of differential transcription of these genes in chromatin. The thermal stability of the heterologous hybrids formed between one of the adult sheep globin cDNAs (α, β^B) and the other adult beta globin mRNAs (α, β^A and α, β^C) have also been determined. Again the T_M of the heterologous hybrids is lower to an extent which reflects a nucleotide sequence difference no greater than that predicted from the amino acid sequence difference of the chains. This small reduction in terminal stability may not allow preparation of specific probes selective for the individual sheep adult beta globin mRNA sequences.

Significance to Biomedical Research and the Program of the Institute: The globin genes provide a unique model for investigating gene regulation in eukaryotic cells. This knowledge will be applied to the study of human disease states, specifically thalassemia, and also to the regulation of the human globin genes.

Proposed Course of the Project: It will be determined whether the individual sheep globin genes (beta and gamma) are regulated primarily at the transcriptional level. If this proves to be true an effort will be made to purify specific protein fractions which activate the individual genes either in chromatin or in nuclei from cells of non-erythroid tissues.

Publications:

1. Wilson, G.N., Steggles, A.W., Kantor, J.A., Nienhuis, A.W., and Anderson, W.F.: Cell-free transcription of mammalian chromatin: Quantitative measurement of newly synthesized globin messenger RNA sequences. J. Biol. Chem. 250: 8604-8613, 1975.

2. Wilson, G.N., Steggles, A.W., and Nienhuis, A.W.: Strand selective transcription of the globin genes in chromatin. Proc. Nat. Acad. Sci. USA. 72: 4835-4839, 1975.

3. Wilson, G.N., Steggles, A.W., Anderson, W.F., and Nienhuis, A.W.: Transcription of chromatin by eukaryotic and prokaryotic polymerases. Methods in Cell Biology (Stein, editor), in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02208-03 MHB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Iron Chelation in Transfusional Hemosiderosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. W. Nienhuis, Chief, Section on Clinical Hematology	MHB, NHLI
OTHER:	W. L. Henry, Senior Investigator	CB, NHLI
	R. A. Rufo, Clinical Associate	BB, CC
	D. T. Peterson, Clinical Associate	MHB, NHLI
	F. C. Bartter, Chief, Endocrinology Branch	NHLI
	Roger Aamodt, Chief, Section on Whole Body Counting	NMD, CC

COOPERATING UNITS (if any)

Cardiology Branch, NHLI; Endocrinology Branch, NHLI; Section on Whole Body Counting, Nuclear Medicine Department, CC

LAB/BRANCH
Molecular Hematology Branch

SECTION
Section on Clinical Hematology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are designed to evaluate the clinical effects of iron chelation agents. Current studies include the evaluation of chronic intensive therapy with desferrioxamine. The medication is given either intravenously with supplemental intramuscular injections, or by chronic subcutaneous infusion. The results of treatment are determined by clinical evaluation, by endocrinological studies, serum ferritin measurements, and echocardiographic observations at regular intervals.

901

PHS-6040
(12-75)

Objectives: The objective of this study is to evaluate available iron chelators, to maximize their effectiveness, and to test new chelators as they become available. An effort is being made to evolve clinical criteria which would be useful to determine the efficacy of chronic chelation therapy.

Methods: Desferrioxamine is administered six times daily by intramuscular injection, by continuous intravenous infusion for 24-72 hrs, or by continuous subcutaneous infusion for three to five day periods. Iron excretion in the urine is measured daily and, at intervals, iron excretion in the stool is determined while the patient is consuming a standard diet of known iron content. Detailed endocrinological evaluation is performed prior to and at regular intervals during chelation therapy. These studies include assessment of pituitary function by measurement of TSH and gonadotropins after administration of appropriate releasing factors. Cardiac function is assessed by standard clinical criteria and by serial echocardiographic observations.

Major Findings:

1. The value of serial echocardiographic observations in patients with iron overload has been further validated. In a series of 40 patients ranging in age from 2 to 25 years, left ventricular wall thickening, cardiac dilatation of variable degree, and increased cardiac mass has been demonstrated. The ejection fraction has been identified as a very important parameter of left ventricular function. It was normal in all patients except four. These others had overt congestive heart failure or developed it within a few weeks and each died within six months.

2. A serum ferritin assay has been established using materials in the laboratory. Serial measurements of ferritin in two heavily iron loaded patients have documented the influence of variables other than iron overload on the absolute value of this parameter. Thus, in one patient it varied directly with SGOT and in another was shown to be elevated during a period of infection.

3. Chronic subcutaneous administration of desferrioxamine in a dose of 1.5 gm/day for five days each week resulted in excretion of 60-100 mg of iron per day in one patient and approximately 50 mg/day in another. Maintenance of this level of excretion for one year would result in unloading of 5-10 gm of iron over and above that amount the patient receives by transfusion. No untoward side effects of the chronic subcutaneous therapy have been observed to date.

Significance to Biomedical Research and to Institute Program: Hemosiderosis is a major cause of morbidity and mortality in patients requiring prolonged transfusion therapy. The role of iron chelators in improving the clinical course of these patients must be ascertained.

Proposed Course of the Project: The project will be continued until a suitable iron chelator is found and evaluated or until the need for transfusion in thalassemia and other congenital hemolytic anemias is removed.

Publications:

1. Arnett, E.N., Nienhuis, A.W., Henry, W.L., Ferrans, V.J., Redwood, D.R., and Roberts, W.C.: Massive myocardial hemosiderosis: A structure-function conference at the National Heart and Lung Institute. Am. Heart J. 90: 777-787, 1975.

2. Nienhuis, A.W., Delea, C.A., Aamodt, R., Bartter, F.C., and Anderson, W.F.: Evaluation of desferrioxamine and ascorbic acid for the treatment of chronic iron overload. National Science Foundation. Original Contribution Series. 1976 (in press).

3. Nienhuis, A.W. and Anderson, W.F.: Treatment of transfusional hemosiderosis with desferrioxamine and ascorbic acid. Symposium on the Development of Iron Chelators for Clinical Use. U. S. Printing Office, 1976. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL02209-03 MHB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cardiac Hemolytic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. B. Weiss, Research Associate	MHB, NHLI
Other:	A. W. Nienhuis, Chief, Section on Clinical Hematology	MHB, NHLI
	S. M. Levenson, Staff Fellow	NMD, CC
	C. L. McIntosh, Senior Surgeon, Clinic of Surgery	NHLI

COOPERATING UNITS (if any)

Cardiac Surgical Branch, NHLI and Nuclear Medicine Department, CC

LAB/BRANCH
Molecular Hematology Branch

SECTION
Section on Clinical Hematology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is the medical treatment of cardiac hemolytic anemia. In order to do this, the disease is being characterized in terms of: 1) severity of hemolysis; 2) ferrokinetics; 3) blood oxygen affinity, and 4) bone marrow function. In addition, a small group of patients with adequate iron stores and functioning valve prostheses are being treated with the androgenic steroid, oxymetholone, in an effort to augment the production of red blood cells.

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PHS-6040
(12-75)

Objectives: The objectives of this study are: 1) to characterize the etiology of the anemia in patients with cardiac valve prostheses; 2) to evaluate various parameters of hemolysis as to their usefulness in measuring hemolytic rate in patients with cardiac hemolytic anemia; and 3) to determine whether oxymetholone, a potent oral androgen, will benefit those patients with non-compensated hemolysis.

Methods: Patients are admitted to the Clinical Center for the following studies: red cell chromium survival, ferrokinetics, bone marrow biopsy, whole blood oxygen affinity, and red cell 2,3-DPG. The charts of all anemic patients who have undergone cardiac valve replacement at the NIH are being reviewed.

Major Findings:

1. A group of patients has been identified with significant anemia despite normal prosthetic valve function and adequate iron stores. They do not show a compensatory reticulocytosis and ferrokinetics demonstrate rapid plasma iron clearance but normal plasma iron turnover and utilization despite accelerated red cell destruction. It is felt that these patients have a defect in red cell production (decreased marrow reserve) as well as hemolysis.

2. One hundred and twenty patients receiving valve replacement at the NIH (10% of total) have developed anemia at least transiently. Traumatic hemolytic anemia is the most common cause (67%).

3. Many patients who develop anemia following valve replacement had abnormal erythrocyte sedimentation rates preoperatively, suggesting a subclinical inflammatory process which might compromise bone marrow function.

4. Of the two patients treated with oxymetholone to date, one has had a good response and one has not responded.

Significance to Biomedical Research and to the Institute Program: Cardiac hemolytic anemia is a significant complication of prosthetic valve replacement. Treatment by reoperation to decrease hemolysis has high morbidity and mortality. Other ways to minimize the severity of the anemia are being studied.

Proposed Course of the Project: Additional patients are being recruited to study the effect of oxymetholone on the severity of anemia in patients with hemolysis. The patients currently followed by the Molecular Hematology Branch will be re-evaluated periodically in an effort to identify factors responsible for the inadequate bone marrow response to hemolysis.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL02210-02 MHB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Regulation of the Respiratory Function of Blood

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI.:	R. M. Winslow	Senior Investigator	MHB NHLI
OTHER:	M. L. Swenberg	Research Chemist	MHB NHLI
	R. Berger	Senior Investigator	LTD NHLI
	E. Gross	Chief, Sect. Molecular Structure	RRB NICHD
	R. Shrager	Mathematician	LSMM DCRT
	L. Rossi-Bernardi	Prof., Enzymology, University of Milan, Milan, Italy	
	S. Charache	Visiting Scientist, Johns Hopkins University, Baltimore, Md.	

COOPERATING UNITS (if any)
LTD/NHLI; RRB/NICHD; LSMM/DCRT;
University of Milan and Johns Hopkins University

LAB/BRANCH
Molecular Hematology Branch

SECTION
Section on Clinical Hematology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	none

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this work is understanding the molecular and cellular regulation of hemoglobin function. This is approached by the study of abnormal hemoglobins, sheep and goat hemoglobin, whole blood oxygen affinity, and the influence of various cellular components on the oxygen affinity of pure hemoglobin. In the past year a report of Hemoglobin McKees Rocks, a mutant with exceedingly high oxygen affinity has been completed, and the measurement of the oxygen dissociation curve of normal human blood has been accomplished. The precision of the latter data is of sufficient quality that for the first time computer analysis of blood oxygenation data can be carried out in order to evaluate the relation between the function of pure hemoglobin and red cells.

PHS-6040
(12-75)

Methods: The methods used in this work are the result of cooperative efforts between the Molecular Hematology Branch, National Heart and Lung Institute, the Laboratory of Technical Development, National Heart and Lung Institute, and the Cattedra Di Enzimologia, University of Milan.

1. The automatic continuous oxygenation apparatus used for the measurement of whole blood oxygen affinity by the addition of hydrogen peroxide, has been developed further. The method is now nearly completely automated and the data are completely analyzed by computer. A more refined version of the cuvette has been designed by LTD and should be in operation by July 1, 1976. An improved electrolyte solution for the oxygen electrode has been contributed by the Milan Group and the method is now fully operational and in daily use, both for research and clinical purposes.

2. New mixing methods have been devised for the precise measurement of the extreme top and bottom ends of whole blood oxygen dissociation curves. This method allows even greater precision than that available previously, and for the first time, a complete oxygen dissociation curve from 0 to 650 mm mercury can be obtained in less than two hrs. after venesection. The results of this analysis will replace previously accepted normal curves.

3. Analysis of the structure of Hemoglobin McKees Rocks was completed during the past year and the methods for rapid screening of mutant human hemoglobins has been developed to the point of being nearly routine.

Findings:

1. A fusion peptide of beta and delta chains of human hemoglobin was analyzed and found to be Hemoglobin Lapore.

2. The oxygen dissociation curve for normal human blood has been measured to greater precision than that previously possible. The combination of the hydrogen peroxide method for whole blood oxygen dissociation curves mentioned above and the new mixing methods were combined to achieve this end. This data has been analyzed in conjunction with Dr. Shrager of the DCRT in order to obtain a fit of this data to various existing models of hemoglobin function. It appears that whole blood oxygenation curves can be analyzed in terms of the models used for pure hemoglobin solution. Therefore, the influence of cellular factors can be evaluated with respect to such models.

Significance to Biomedical Research and the Program of the Institute: The delivery of oxygen to tissues is the primary respiratory function of blood. The present studies can further our understanding of the adaptive mechanisms which operate when this function is compromised, such as anemia, respiratory disease, and abnormalities of the red cell which lead to altered oxygen affinity. Moreover, such understanding could lead to new therapeutic possibilities.

Proposed Course of the Project:

1. A systematic evaluation of the effects of various cellular factors, such as pH, 2,3-DPG, CO_2, hemoglobin concentration, and temperature will be carried out using the techniques which have now been successfully used in the

measurement of the normal whole blood oxygen dissociation curve. At each step, the data will be compared with that obtained for pure hemoglobin solutions in an effort to determine whether the properties of whole blood are the sum of the individual properties of the isolated components.

2. As this work proceeds, quantitative data from computer analysis will be accumulated; the ultimate goal will be to describe the various influences of blood oxygen affinity from the known quantitative effects of each of the cellular components. Finally, this body of data will be integrated with physiologic information about pulmonary function and tissue oxygen delivery to attempt to build a working model of the respiratory function of blood.

3. Oxygen delivery and blood oxygen function in the goat will be continued in the next year with the addition of Dr. Miki Samaja to our Section. Using the preliminary observation that goat and sheep hemoglobins are extraordinarily sensitive to CO_2, (possibly because of unusual alterations at the amino terminus of the beta chains in Hb C) will be studied. It is our intention that work on the goat system will parallel that in the human system and that ultimately the goat model can be developed and integrated with physiologic information on oxygen delivery in the goat to aid in the understanding of the physiologic and evolutionary significance of the switch to Hb C during conditions of anemia or high altitude hypoxia.

Publications:

1. Winslow, R.M., Swenberg, M.L., Gross, E., Chervenick, P.A., Buchman, R.R., and Anderson, W.F. Hemoglobin McKees Rocks: A human "nonsense" mutation leading to a shortened β-chain. The J. of Clin. Invest. 57: 7720781 (March 1976).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02205-03 MHB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanism of Action of the Enzyme RNA-directed DNA Polymerase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. B. Weiss	Research Associate	MHB NHLI
	L. J. Krueger	NIH Predoctoral Fellow	MHB, NHLI
OTHER:	W. F. Anderson	Chief	MHB, NHLI
	W. C. Merrick	Research Chemist	MHB, NHLI

COOPERATING UNITS (if any)

Viral Oncology Branch, NCI

LAB/BRANCH

Molecular Hematology Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study the mechanism of RNA-directed DNA polymerase. The topics of present interest are: 1) the effects of template base composition on transcription; 2) the kinetics of transcription with emphasis on inhibition studies to determine the order of substrate addition; 3) preparation of full-size cDNA to be used in other projects.

PHS-6040
(12-75)

Objectives: The enzyme RNA-directed DNA polymerase, also known as reverse transcriptase, from avian myeloblastosis virus provides a means for synthesizing a DNA gene directly from isolated messenger RNA. Unfortunately, the quality and quantity of the DNA product are variable, depending on which RNA is used as template. It is desired, therefore, to learn the mechanism of action of the enzyme in order to establish optimal conditions whereby a complete (and active) DNA gene can be transcribed from any RNA.

Methods: The enzyme is purified by standard procedures from avian myeloblastosis virus obtained through the National Cancer Institute. Globin mRNA is isolated from human, rabbit and mouse reticulocytes; DNA product analysis is by formamide-polyacrylamide gel electrophoresis. Polyribonucleotides (random copolymers and block copolymers) are bing synthesized by means of various enzymatic and chemical techniques including use of the enzyme polynucleotide phosphorylase.

Major Findings:

1) RNA-directed DNA polymerase transcribes the various oligonucleotides differently. Using oligo(I) of size 2.5 S as template and $(dC)_{10}$ as primer, a large (>22 S) product was formed. Similarly, using $(A)_{20}$ as template and $(dT)_{10}$ as primer, a large (10-16 S) product was formed. However, using $(C)_{13}$ as template and $(dG)_8$ as primer, the oligo(dG) product coelectrophoresed with the template. No significant activity was obtained with oligo(U) templates. Thus, there is slippage with oligo(I) and oligo(A), faithful transcription with oligo(C), and poor transcription with oligo(U).

2) The size of the DNA product synthesized by RNA-directed DNA polymerase is important in cDNA-mRNA hybridization reactions. The size is influenced by the reaction conditions. Using rabbit globin mRNA as template, the cDNA product contains some full-sized material when synthesis is done at high (>50 μM) deoxynucleoside triphosphate concentrations. The concentration and size of oligo(dT) used as primer has no effect while increasing the concentration of mRNA used as template causes the cDNA to contain more small material. The larger cDNAs had a higher T_M when hybridized to globin mRNA, a lower dTMP/dCMP ratio (indicating that the poly(dT) region constituted a smaller fraction of the molecule) and gave increased protection of ^{125}I-labeled mRNA from S1-nuclease digestion. The full-sized cDNA was especially useful for studying the RNA transcribed from chromatin by RNA polymerase; RNA transcribed from rabbit bone marrow chromatin by E. coli RNA polymerase hybridized twice as efficiently to full-size cDNA as it did to partial cDNA. This suggests that the RNA transcription, although incomplete, is not randomly initiated.

3) Using highly purified RNA-directed DNA polymerase at low concentrations and long incubation times, the enzyme was found to synthesize more than one DNA molecule per enzyme molecule (7 with globin mRNA as template, 115 with poly(C). This demonstrates that the enzyme can reinitiate and thus, is amenable to Michaelis-Menton type kinetic analysis.

4) The four random copolymers containing inosine are all active as template. Poly(I,G) is unique amongst the random copolymers in that it base pairs perfectly with primer. Compared with poly(I), poly(I,G) is only 1/6 as effective as template, having both a higher K_M and a lower V_{max}. The product has two peaks on SDS-polyacrylamide gel; one, accounting for 23% of the material coelectrophoreses with the 3.6 S template while the second is much larger (10-18 S). The other three copolymers are also transcribed less than poly(A), poly(C) or poly(I) but more than with poly(U).

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Significance to Biomedical Research and Institute Program: The ability to synthesize a DNA gene in vitro is a major step towards the goal of successful therapy of human genetic diseases. The role ofzthe enzyme itself is important in understanding the pathogenesis of some cancers.

Proposed Course of the Project: Detailed studies on the mechanism of action of the enzyme RNA-directed DNA polymerase are being carried out.

Publications:

1. Falvey, A.K., Weiss, G.B., Krueger, L.J., Kantor, J.A. and Anderson, W.F.: Transcription of single base oligonucleotides by ribonucleic acid-directed deoxyribonucleic acid polymerase. Nucleic Acids Res. 3, 79-88, 1976.

2. Krueger, L.J., Weiss, G.B., Merrick, W.C., Lloyd, M.A., and Anderson, W.F.: Cyclingzof RNA-directed DNA polymerase on natural and synthetic RNA templates. Nature 260, 363-365, 1976.

3. Weiss, G.B., Wilson, G.N., Steggles, A.W., and Anderson, W.F.: Importance of full-size complimentary DNA in nucleic acid hybridization. J. Biol. Chem. 251 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02207-03 MHB

PERIOD COVERED
July 1, 1975 - June 30, 1976

TITLE OF PROJECT (80 characters or less)

Globin Gene Expression in Somatic Cell Hybrids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Arthur Nienhuis
W. French Anderson

OTHER: Jane Barker
Ann Huang
Daphne Kamele
Albert Deisseroth (Boston)

COOPERATING UNITS (if any)

Department of Biology, Yale University

LAB/BRANCH
Molecular Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart and Lung Institute, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the regulation of globin gene expression. The approach is to study somatic cell hybrids between mouse erythroleukemia cells and established cell lines. The presence or absence of the globin genes as well as the presence or absence of globin mRNAs is correlated with chromosome composition of the hybrid.

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Objectives: Somatic cell hybrids, generated by fusion of erythroid cells and established cell lines, have been used to obtain continuously replicating cells capable of globin synthesis. These have then been utilized to study regulation of expression of the globin genes. In addition, human-mouse hybrids containing one or more human chromosomes have been studied in order to attempt to localize the human globin structural genes.

Methods: Erythroid cells are obtained from human and animal bone marrow, mouse spleen, or fetal liver and purified by differential immune lysis, buoyant density centrifugation, or unit gravity sedimentation. These cells are fused to established cell lines using inactivated Sendai virus. Selective media are constructed which favor the growth of the hybrid cells over the parent lines and the hybrid nature of the resulting clones is confirmed by analysis of the chromosomal composition and isozyme content. Globin messenger RNA is detected by hybridization of the hybrid cell RNA to appropriate complementary DNA (cDNA) probes and the presence of globin is detected by radioactive leucine incorporation followed by column chromatography. Gene localization studies require DNA-cDNA hybridization using fractionated and species specific globin cDNA probes.

Major Findings:

1) Somatic cell hybrids, obtained from fusion of human marrow cells with Friend mouse erythroleukemia (MEL) cells, exhibited either: co-expression of human and mouse globin, expression of mouse globin only, or total absence of globin gene expression. These hybrids, containing a partial complement of human chromosomes, were used to demonstrate that the human alpha and beta globin genes are located on different chromosomes. A mouse x human fibroblast line contained the human alpha but not the beta globin gene as shown by DNA-cDNA hybridization; while a human marrow x mouse erythroleukemia line expressed the human beta but not the alpha globin gene as shown by mRNA-cDNA hybridization.

2) In order to identify the specific chromosomes bearing human globin genes, a number of mouse-human hybrids are being tested for the presence of the human globin gene using sufficiently stringent conditions for DNA-cDNA hybridization such that human and mouse sequences do not cross hybridize. The chromosomal composition of each hybrid cell population, at the time the DNA is extracted for globin gene identification, is being determined by a combination of techniques including Giemsa, Q, and C banding, Giemsa 11 staining, and isozyme analysis. Correlation of chromosomal content with globin gene content should indicate which human chromosome carries the alpha and which carries the non-alpha globin genes. Thus far, of the 9 hybrids tested, 3 possess both the alpha and beta globin genes, 3 possess only the alpha, and 3 possess neither gene. Karyological analysis is not yet complete.

3) MEL x human fibroblast hybrids are being used as a model system to examine regulation of globin gene expression. Three hybrid cell lines were incubated in 2% dimethylsulfoxide, an agent which induces hemoglobin synthesis in the parental erythroleukemia cells. Neither human nor mouse globin mRNA sequences could be detected by mRNA-cDNA hybridization. Thus, loss of the genes by chromosomal segregation did not account for their failure to be expressed. Co-cultivation of the mouse erythroleukemia cells with excess human fibroblasts did not prevent erythroid differentiation of MEL cells in the presence of DMSO. Likewise, co-cultivation of human fibroblasts in the presence of excess MEL cells did not induce globin synthesis in the human fibro-

blasts. Globin gene expression was preserved in tetraploid cells generated by fusion of two erythroleukemia lines. Thus extinction of globin gene expression in the MEL x human fibroblast hybrids occurred at the level of mRNA production and appeared to be due to the presence of the fibroblast genome within the hybrid cells.

Significance to Biomedical Research and Institute Program: An understanding of the mechanism of mammalian gene regulation is of fundamental importance in understanding human genetic disease. This program offers an opportunity for insight into genetic regulation by exploiting our knowledge of the expression of the globin genes in somatic cell hybrids.

Proposed Course of the Project: The studies leading to the chromosome assignment of the alpha and beta globin structural genes will be continued. Somatic cell hybrids which coexpress both mouse and human globin chains will be studied to attempt to learn the factors controlling expression of the globin genes.

Publications:

1. Deisseroth, A., Barker, J., Anderson, W.F., and Nienhuis, A.: Hemoglobin synthesis in somatic cell hybrids: II. Coexpression of mouse with human or Chinese hamster globin genes in interspecific somatic cell hybrids of mouse erythroleukemia cells. Proc. Nat. Acad. Sci. U.S.A. 72, 2682-2686, 1975.

2. Deisseroth, A., Velez, R., and Nienhuis, A.W.: Hemoglobin synthesis in somatic cell hybrids: Independent segregation of the human alpha- and beta-globin genes. Science 191, 1262-1264, 1976.

3. Deisseroth, A.: Isolation of hybrid cells that exhibit markers of erythroid differentiation. New Engl. J. Med. 294: 148-152, 1976.

4. Anderson, W.F., Deisseroth, A.B., Velez, R., Nienhuis, A.W., Ruddle, F.H., and Kucherlapati, R.S.: A new technique for mapping the human hemoglobin genes. In Human Gene Mapping 3 (V.A. McKusick, ed.). National Foundation March of Dimes (in press).

5. Deisseroth, A., Velez, R., Burke, R., Minna, J., Anderson, W.F., and Nienhuis, A.: Extinction of globin gene expression in human fibroblast X mouse erythroleukemia cell hybrids. Somatic Cell Genetics (in press).

Annual Report of the
Pulmonary Branch
National Heart and Lung Institute
July 1, 1975 through June 30, 1976

The major function of the lung is to exchange gases between the atmosphere and the blood. To accomplish this, the lung has evolved as a complex structure which brings together the atmosphere and the blood in a fashion which is finely regulated to insure maximum efficiency. The major thesis of this laboratory is that the process of gas exchange is critically dependent on lung cells in their ability to maintain lung structure and function.

The adult lung consists of approximately 40 cell types and a complex extracellular matrix composed of collagen, elastic fibers and proteoglycans. These complexities make the application of biochemical methodologies to the study of lung structure and function extremely difficult, since each of the cell types and extracellular matrix components contribute to biochemical measurements of the organ as a whole. In addition, the lung is continuously bombarded by mechanical, neural and endocrine factors which attempt to modulate the inherent properties of lung cells and their surrounding matrix. Thus, in order to define the lung on a biochemical basis, it is necessary to simplify by significantly reducing the number of variables that must be dealt with. In general, two methodologies have made the biochemical approaches to study of lung structure and function possible.

First, each of the major components of lung structure (parenchyma, blood vessels, airways) can be dissected from lung, cut into small pieces and maintained in explant culture without serum for at least 48 hours with no significant change in the differentiated state. Second, several of the major cell types of lung can now be isolated and cultured for long periods of time.

These two systems have enabled us to carefully define the composition, synthesis and degradation of the major connective tissue components of lung with particular emphasis upon collagen. Their use has been readily extended to studies on the sub-cellular level using cell-free protein synthesis and classic enzymologic methods. The following is a summary of the ongoing projects in the Pulmonary Branch for this past year.

I. Models of Lung Growth

Two models are used to investigate the control of connective tissue synthesis and degradation during lung growth: (1) changes during normal neonatal lung growth; and (2) "compensatory" lung growth following unilateral pneumonectomy.

Prior studies have defined the normal amounts of collagen and rates of collagen synthesis in lung in both models. The molecular basis of the controls in collagen synthesis have been partially defined using cell-free systems to quantitate collagen messenger RNA bearing RNA polysomes during neonatal lung growth. During the past year our emphasis has shifted toward

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evaluating changes in glycosaminoglycans during neonatal lung growth and to correlating morphological and biochemical changes in post-pneumonectomy lung growth.

In the rabbit, parenchymal glycosaminoglycan content ranges from 0.2 to 0.4% (wt/dry wt), with highest concentration in adult lung. There are significant changes in types of glycosaminoglycans present at different ages. Fetal lungs contain a relatively high proportion of chondroitin 4-sulfate, while the glycosaminoglycans in lung parenchyma of older animals was predominantly dermatan sulfate, heparan sulfate and heparin. The rate of synthesis of total glycosaminoglycans per cell increased with age until the weanling period and then fell to low rates of synthesis in mature rabbits. Fetal rabbit lung parenchyma synthesized mostly hyaluronic acid and heparan sulfate while in weanling rabbit parenchyma, hyaluronic acid and chondroitin 4/6 sulfate synthesis was greatest. In mature animals the rates of synthesis of all types of glycosaminoglycans was relatively low, but there was relatively greater emphasis on synthesis of dermatan sulfate and heparin.

Preliminary morphometric data of post-pneumonectomy lung growth suggests that one month after pneumonectomy the remaining lung doubles in size with this doubling being associated with the doubling of the number of alveoli. There appears to be no abnormalities in alveolar ducts, parenchymal tissue thickness, or other parameters of alveolar size. Thus, it appears that post-pneumonectomy lung growth in the rabbit is remarkably similar to normal neonatal lung growth in the same animal.

These models provide a means of studying the lung during periods when connective tissue synthesis and degradation are changing. Utilizing investigations at the cellular and sub-cellular levels, it should be possible to understand the control of structural and non-structural protein synthesis in lung. This has significant applications in the control of fibrosis in the interstitial lung disorders and for the replacement of alveoli in the emphysematous disorders.

II. Isolation, Culture and Characterization of Lung Cells

The use of biochemical technology to understand human lung disease is limited by the complexity of the organ, the unavailability of large quantities of lung cells, and the multitude of variables involved in attempting to study the lung in situ. Even though the biochemist can approach the study of lung cells with a vast armamentarium of concepts and sophisticated methodology, the inherent complexity of dealing with multiple cell types immediately hinders the sorting out of the contribution of each cell type to the biochemical parameters being measured. In addition to the problems of cell purity, the study of lung cells in situ is compounded by the multitude of factors which impinge on each cell type to attempt to vary inherent properties. Thus, to reduce the number of (mostly unknown) variables to a minimum, it has become necessary to develop techniques by which individual populations of lung cells can be isolated, cultured and studied. One of the primary objectives of this laboratory is to develop the technology to culture the cells from the lung that are important in the maintenance of the

normal parenchymal structure in health and disease. Of particular interest are the processes by which these cells synthesize and degrade the extracellular connective tissue matrix of the alveolus.

It is now possible to isolate and/or culture several cell types from lung and the following types have been utilized by this laboratory: (1) alveolar macrophages from the adult human and adult rabbit; (2) fibroblasts from the adult human lung, fetal human lung, newborn rabbit lung, newborn rat lung, fetal cat lung, and adult rabbit skin; (3) endothelial cells and smooth muscle cells from human umbilical vein; (4) epithelial cells from the fetal cat lung; and (5) alveolar type II cells from the adult rabbit lung. These cells have been utilized in the following studies:

(1) Methods have been developed to quantitate the absolute rates of collagen synthesis and degradation in cell cultures. In addition, it is now possible to quantitate the relative amounts of type I and type III collagen synthesized by these cells.

(2) Using molecular weights maps, it has been determined that the human fetal lung fibroblast secretes the same proteins at passage 3 and passage 15. The rates of collagen synthesis per cell are identical as are the rates of total non-collagen protein synthesis throughout this time period.

(3) Methods used to quantitate the types of glycosaminoglycan synthesized and secreted by cells are being utilized to compare the differentiated properties of lung epithelial cells with alveolar mesenchymal cells. Preliminary studies suggest they secrete different types of glycosaminoglycans.

(4) Molecular weight maps are utilized to characterize different types of lung cells. The patterns of secreted proteins of the alveolar type II cell, monocyte, alveolar macrophage and fibroblast are completely different. These maps are characteristic of these cell types and can be used in addition to morphology to specify the types of cells in culture.

(5) Evaluation of collagen synthesized by lung cells in culture shows that a significant proportion of the collagen synthesized is degraded, probably intracellularly. Methods have been developed to quantitate this process and evaluation is ongoing to attempt to manipulate the intracellular collagen degradation.

(6) Skin fibroblasts are utilized to evaluate, diagnose and define the hereditary disorders of connective tissue. This data is being combined with information of connective tissue in lung parenchyma and with sophisticated studies of pulmonary function abnormalities in these disorders. By this means, an understanding of the biochemical-mechanical correlates of lung function are being achieved.

(7) Studies with cultured cells have suggested that the glycosylation of collagen synthesized in culture is markedly different from that of characteristic collagens found in these organs. This implies that either

there are significant post-translational mechanisms which modulate collagen carbohydrates after their synthesis or that new, as yet undescribed, collagens are being synthesized by these cells in culture.

(8) Cell cultures from patients with fibrotic lung disease and normal humans are being utilized in cell-cell interaction studies involving immune-competent components from patients with fibrotic lung diseases. For example, lymphocytes from patients with pulmonary fibrosis are placed on fibroblasts labeled with ^{51}Cr to demonstrate that these lymphocytes induce lysis of the fibroblasts. These cell-cell models are being further investigated to evaluate pathogenic mechanisms of the inflammatory and immune systems in the fibrotic lung disorders.

(9) Patients with pulmonary fibrosis are usually biopsied at one time in the clinical course. These biopsies are being utilized to culture lung cells from these patients. These cells are used in preliminary studies and are being frozen away for recall as methods develop.

(10) Cultured lung cells are being utilized in cell-cell interaction studies from models of pulmonary fibrosis. For example, pulmonary alveolar macrophages are being fed silica or other environmental pollutants and the supernatant from these cultures are being evaluated for the effect on the differentiated state of lung cells.

The mechanisms by which the differentiated state is controlled in lung are the mechanisms by which the lung maintains its structure and function. The establishment of diploid cell lines of the cells responsible for the maintenance of alveolar structure and function in health and disease allows investigation at the molecular level. This includes identification of normal mechanisms, the maintenance of lung and how these mechanisms are varied following lung injury.

III. Accumulation of Collagen in Human Lung in Health and Disease

The connective tissue of lung is fundamental for lung structure and mechanical properties. In the interstitial lung disorders, many of which result in pulmonary fibrosis, this connective tissue structure is disordered such that gas exchange is severely hampered. With the ultimate aim being an ability to control these abnormalities through molecular mechanisms, our laboratory is developing the technology to quantitate human lung collagen composition, synthesis and degradation.

Prior studies have established the values for concentration of collagen and rates of collagen synthesis in control lungs. Studies with 8 patients with idiopathic pulmonary fibrosis and 4 patients with pulmonary fibrosis secondary to tumors (compared to 6 controls) have shown: (1) the concentration of collagen (amount per unit dry weight) in all three groups are identical; and (2) the rate of collagen synthesis per cell (averaged over all cells in biopsy) are identical. Quantitation of multiple (40-50/lung) biopsies of post-mortem specimens of lungs of patients with idiopathic pulmonary fibrosis compared with normal (death due to non-lung causes) controls have demon-

strated that the lack of differences between the collagen concentration in idiopathic pulmonary fibrosis versus controls is not due to a "sampling problem" since the distribution of values obtained from the post-mortem lungs was the same in both groups.

In idiopathic pulmonary fibrosis, there is "scar" in the alveolar interstitium. In biochemical terms, "scar" or "fibrosis," means collagen deposition in excess amounts, abnormal type and/or location. Through the techniques developed in our laboratory, it has become possible to: (1) quantitate the concentration of collagen in biopsy specimens of human lungs; and (2) quantitate the rate of collagen synthesis (averaged over all lung cells) in lung biopsies. The surprising results of these studies strongly suggest: (1) there is no increase in collagen concentration in idiopathic pulmonary fibrosis; and (2) there is no difference in the rates of collagen synthesis in this disease compared to normal. Interestingly, patients who have pulmonary fibrosis secondary to tumors in their lungs have similar findings. In comparison, rabbits who have experimentally induced radiation fibrosis have significant elevations of collagen concentration preceded by elevations in rates of collagen synthesis. Thus, we have to rethink the biochemical concepts of the "fibrotic" disease state in human lung. Idiopathic pulmonary fibrosis is not simply a disease in which the lung makes more collagen, it is probably a much more complex process. For example, idiopathic pulmonary fibrosis may be associated with: (1) a relative increase in the population of cells that synthesize collagen; (2) a shift in the types of collagen synthesized; (3) a decrease in the rate of collagen proteolysis; or (4) a localized derrangement where collagen is synthesized or laid down. These concepts also have application to the emphysematous disorders where there is destruction of the connective tissue comprising the lung with either absent or ineffective remodeling of the connective tissue.

IV. Experimental Models of Fibrotic Lung Disease

The major goal of this laboratory is to understand and eventually control the mechanisms involved in connective tissue synthesis and degradation in human lung diseases involving these components. Because the availability of human lung biopsy material is limited, it necessitates that any biochemical techniques applied to this tissue is worked out first in model systems, thus insuring the maximum amount of information and the maximum benefit to the patient. For that reason two groups of models of fibrotic disease are being developed and evaluated: (1) animal models; and (2) tissue culture models.

In the animal models, studies have demonstrated the right lung of irradiated animals (3000 and 6000 R to the right lung) show significant increases in collagen content 8 to 10 weeks after irradiation. This collagen accumulation is paralleled by increases in the rates of collagen synthesis per cell. Morphological studies show cellular injury (particularly the enodthelial cell) prior to the increase in rates of collagen synthesis or accumulation of collagen. These findings are significantly different from similar quantitative measurements in biopsy specimens from patients with idiopathic pulmonary fibrosis.

In tissue culture models, studies with fibroblasts from rabbit and human lungs have demonstrated that approximately 4 to 5% of the total proteins synthesized are collagen. This finding has been variant during the life of the cell (e.g., passage 5 compared to passage 15) as long as the status of cell density (e.g., log vs. confluent phases of growth) and intracellular pools of amino acids are considered. Preliminary studies have suggested that comparing the confluent phase of culture to the log phase of culture may be a model system in which to study changes in the control mechanisms governing the types of collagen synthesized by lung cells. The modulation of these controls by inflammatory and immune mechanisms is still in the developmental stage.

As we learn to define the animal and tissue culture models of fibrotic lung disease, we should be able to understand the sensitive control points where we can interrupt the fibrotic process with pharmacological agents.

V. Heterogeneity of Lung Collagen

Collagen is the most abundant protein in lung comprising 10 to 20% of the adult lung by dry weight. There are more than 40 cell types in lung including mesenchymal cells, epithelial cells, endothelial cells and smooth muscle cells all of which have been implicated to synthesize collagen. The primary objective of these studies is to identify the heterogeneous types of collagen in lung and the cells that synthesize it.

The lung parenchyma is composed of at least Type I collagen $[\alpha 1(I)_2 \alpha 2]$ and Type III collagen $[\alpha 1(III)]_3$. Because lung has the morphological evidence of extensive basement membrane, it is likely that basement membrane collagen is present in the lung parenchyma as well. However, studies quantitating 4-hydroxyproline to 3-hydroxyproline in the parenchyma of adult human lung have revealed ratios of approximately 100. Therefore, if basement membrane lung is similar to basement membrane from other organs (kidney basement membrane has very large quantities of 3-hydroxyproline compared to 4-hydroxyproline), then less than 1% of the lung parenchyma is basement membrane. It is also possible that lung basement membrane is different than kideny basement membrane in which new markers will have to be developed for its evaluation and quantitation.

The tracheo-bronchial tree contains Type II collagen (cartilage collagen) and the major blood vessels contain at least Type I collagen and probably Type III collagen as well.

It is now possible to qunatitate the types of collagen synthesized by lung cells in culture, and methods are being evaluated to quantitate types of collagen synthesized by lung explants in vitro. With these methods, it should be possible to soon quantitate the relative type of different collagens being synthesized in the lung parenchyma in health and disease. In the tissue culture systems, these methods are adaptable to understanding the molecular control of the synthesis of these collagen types and how this control might be modulated when these systems go awry in lung disorders.

An understanding of the mechanisms of control of collagen synthesis in lung will help in understanding of the pathological processes involving collagen in these disease states. For example, since the fibrotic lung disorders have many primary stimuli including inhalation of toxic materials, hypersensitivity states and radiation injury, it is likely that these disorders are associated with injury to different lung cells. This suggests the possibility that there may be a heterogeneity in the types of collagen synthesized in the different lung diseases. It may be possible to classify the interstitial lung disorders on the basis of the types of collagen synthesized in a similar fashion to the lipoprotein disorders and certain hemaglobin disorders. In addition, the heterogeneity of lung collagen may be a crucially important determinant of lung mechanical properties in the normal development of lung.

VI. Studies of Patients with Fibrotic Lung Disease

The fibrotic lung disorders represent 15-20% of the non-infectious disorders of the lung. There are more than 130 separable disease entities associated with pulmonary fibrosis, but they can be categorized into ten general groups (occupational and environmental entiolgies, drug reactions, physical agents, infectious disorders, secondary to cardiac disease, pulmonary vascular disease, neoplastic disease, congenital and familial, unknown etiology but with characteristic morphology, idiopathic pulmonary fibrosis). The Pulmonary Branch, NHLI, has undertaken a detailed study of patients with pulmonary fibrosis, particularly those with environmental etiologies and those with idiopathic pulmonary fibrosis (IPF). The latter represents a chronic, devastating illness, invariably resulting in death an average of 4 to 5 years from the onset of symptoms. Although approximately 5-10% of patients with IPF respond to corticosteroids, there is no known treatment for the remainder. The objectives of our studies are as follows: (1) to determine the etiology of IPF; (2) to follow patients with IPF longitudinally to determine (a) the natural history of the disorder and (b) which pulmonary function parameters are most sensitive to the disease process; (3) to correlate pulmonary alveolar constituents (alveolar lavage fluid and cells) in IPF with histopathology and pulmonary function parameters as well as with alveolar constituents in other fibrotic disorders; (4) to correlate radioisotopic monitors of lung function (ventilation, perfusion and gallium scans) with the disease process; (5) to correlate the histopathology in IPF with biochemical and functional parameters; (6) to study the therapy of IPF; (7) to study abnormal inflammatory and immunologic processes associated with the continued pathogenesis of the IPF; and (8) to study genetic determinants of IPF.

Completed studies in patients with IPF have demonstrated: (1) obstruction to airflow in the bronchiole is a significant part of the functional abnormalities in this disorder; (2) the maximum transpulmonary negative pressure necessary to expand the lungs correlates well with the degree of interstitial fibrosis; (3) the rate of decline of the arterial oxygen tension with increasing levels of exercise correlates with the extent of decrease shown histologically; (4) immune abnormalities associated with the collagen-vascular disorders are not frequently found in IPF; (5) ventilatory and

arterial blood gas parameters with exercise are probably the most sensitive monitors of the disease process; (6) patients with IPF have marked abnormalities in ventilation-perfusion mismatching; (7) although the histopathology suggests increased amounts of collagen in the parenchyma of these patients, there is no biochemical evidence that confirm this impression. In fact, the biochemical data demonstrates normal concentrations of collagen in the IPF parenchyma; (8) the rates of collagen synthesis/cell in biopsy specimens from patients with IPF are similar to control patients; (9) broncho-alveolar lavage fluid from patients with IPF (compared to normals) demonstrates a greater percentage of polymorphonuclear leukocytes and eosinophils, elevated levels of IgG but similar levels of the C4 and C6 components of complement. In comparison, patients with chronic hypersensitivity pneumonitis (CHP) have mostly lymphocytes in their broncho-alveolar lavage fluid. The respiratory lymphocytes in CHP have a T-cell to B-cell ratio of 15 to 1 (compared to their own peripheral blood T/B ratio of 3) suggesting the lung can act as an independent immune organ in this disease; (10) ^{67}Gallium-citrate is taken up in the lungs of patients with IPF (65-70%) compared to controls (<5%). The amount of ^{67}Ga uptake correlates with the broncho-alveolar lavage and the histopathologic findings; and (11) peripheral blood lymphocytes from >95% of patients with IPF recognize collagen as antigenic (compared to 0% of normals). In response, these lymphocytes produce migration inhibition factor and will lyse collagen-coated sheep red blood cells. Using anti-human-T-cell antiserum, it has been possible to demonstrate that the T-lymphocyte is necessary to mediate these phenomena.

Preliminary studies in patients with IPF suggest: (1) peripheral lymphocytes from these patients will lyse human lung fibroblasts grown in culture; and (2) tissue typing studies suggest that most patients with IPF have twice normal incidence of HLA-12, an antigen of the B histocompatibility locus.

The fibrotic lung disorders are almost uniformly fatal and affect a significant proportion of the population. Up to this time, there has been no information on pathogenesis and there is essentially no cure. By combining studies of patients with these disorders with our large basic research program concerning the control of connective tissue composition and accumulation in lung, we expect to make major inroads into understanding and eventually curing these disorders.

VII. Investigation of the Hereditary Disorders of Connective Tissue

The connective tissue of lung is complex, consisting of four genetically distinct types of collagen, elastic fibers (elastin together with microfibrils) and seven types of glycosaminoglycans with their associated proteins. This complexity together with the intricate anatomical arrangement of lung parenchyma makes the study of the influence of connective tissue on the control of lung structure and function extraordinarily difficult. The major objectives of this proposal are attempts to use "experiments of nature" involving connective tissue such that if we can understand the biochemical abnormalities of connective tissue in these hereditary disorders, we can then make inferences on the role each connective tissue component plays in

the maintenance of lung structure and function. The lung is the only organ of the body where it is relatively easy using the sophisticated methodology of pulmonary function testing to make direct inferences on the role of connective tissue in controlling lung mechanical properties. Thus, the lung affords an opportunity for understanding the biochemical-mechanical correlates of connective tissue.

Specifically, the ongoing projects in this work are: (1) the development of tissue culture models for studying collagen synthesis in fibroblasts from patients with the hereditary disorders of connective tissue; (2) definition of the quality of collagen synthesized by fibroblasts in culture, e.g., relative hydroxylation of lysine residues, glycosylation of hydroxylysine, relative hydroxylation of proline residues, cross-links, types of collagen synthesized, and quantitation of collagen degradation; (3) to utilize methods to evaluate the types of glycosaminoglycans synthesized in culture as well as the rates of synthesis and degradation of these compounds; (4) to quantitate the rates of synthesis of elastin in these cultures and its associated cross-links; (5) to utilize these methods to specifically define the biochemical abnormalities in patients with disorders such as Marfan's syndrome, Ehlers-Danlos syndrome and Osteogenesis Imperfecta; and (6) to evaluate the same patients using sophisticated methods of pulmonary function including lung volumes, flow rates, body plethysmography, exercise studies with gas exchange parameters, closing volumes, flow-volume curves, maximum flow-static recoil curves, and ventilation-perfusion analysis with nuclear scanning methods.

This is a long term project but with the methodology available today, it should be possible to specifically define the role of each connective tissue component in lung and demonstrate how these components describe pulmonary abnormalities in these hereditary disorders.

This work is just beginning and the past year has been spent primarily in developing methodologies to define the biochemical abnormalities of fibroblasts in patients with these disorders. The first question to be answered is whether fibroblasts derived from patients of different ages behave similarly in culture. Newly synthesized collagen of fibroblasts from seven normal controls ages 20 to 70 were virtually identical with respect to the amount of collagen synthesized, lysine hydroxylation and hydroxylysine glycosylation. The newly synthesized collagen in these cultures has more hydroxylysine than expected from extracted Types I and III collagen and the hydroxylysine in this newly synthesized collagen was glycosylated to a greater extent than extracted Types I and III collagen. This finding suggests that: (1) other types of collagen (e.g., basement membrane collagen) are being synthesized by fibroblast cultures; (2) newly synthesized collagen differs from extracted collagen, with higher values of hydroxylation and glycosylation in the newly synthesized material which is then altered as collagen is laid down in the connective tissue matrix; or (3) fibroblasts do not reflect normal collagen synthesis. In order to answer these questions, rabbits are being labeled *in vivo* and their collagen is being analyzed to compare newly synthesized collagen *in vivo* with that synthesized by fibroblasts in culture. Basement membrane collagen from rabbits are being pre-

pared for comparison with the material synthesized by fibroblasts in culture.

Preliminary studies with fibroblasts from one patient with Ehlers-Danlos syndrome have demonstrated the relative rate of collagen synthesis compared to the synthesis of all other proteins in the culture is markedly high, even though there are normal quantities of lysine hydroxylation and hydroxylysine glycosylation. Preliminary analysis of skin fibroblasts from patients with scleroderma have thus far demonstrated no abnormalities in the parameters being evaluated.

Pulmonary function studies of these patients are ongoing, but as yet a pattern of disease groups has not been established.

The approaches being used will eventually define the molecular defects in the hereditary disorders of connective tissue. By understanding these defects in the same patients in which abnormalities in mechanical properties of lung function are defined will enable us to make significant inferences on the biochemical-mechanical effects of the complex connective tissue in lung. Hopefully definition of these molecular defects will lead to improved diagnostic methods for genetic counseling, prognosis and eventually to rational therapy of these disorders.

VIII. Degradation of Collagen in Lung

The adult lung continually synthesizes collagen at a rate which represents 2 to 4% of the total proteins being synthesized at one time. However, the total amount of collagen in the adult lung remains constant. Therefore, there must exist mechanisms by which collagen is degraded. The objective of this project is to define, quantitate and learn to modify the mechanisms by which collagen is degraded in the lung.

Collagen is an unusual protein in that when it is in its native triple helcial state there are only three proteases known which will degrade it. One is bacterial collagenase which is obviously not operant in the normal lung. The second is an acid cathepsin which is a lysozomal enzyme (described as yet only in liver) which acts at acid pH. The third is via animal collagenase, a neutral protease which acts in the extracellular milieu to degrade triple helical native collagen. It has a pH optimum in the range of 7 to 8, and it cleaves collagen at one site 75% of the distance from the N-terminus of the triple helical molecule. Once this cleavage has been made, the inherent instability of the collagen molecule is such that the triple helical structure cannot be maintained at body temperature, it denatures, and then other proteases active in the extracellular milieu can degrade the remainder of the molecule.

Although previously uninvestigated as related to collagen, it is also possible that there are intracellular mechanisms of collagen degradation in the same fashion as intracellular mechanisms for degradation of intracellular enzymes. This is an attractive hypothesis since it might result in a mechanism by which the cell can control the destruction of "defective" collagen prior to its secretion.

The objectives of this project are to define the collagenases present in lung and to determine if intracellular mechanisms of collagen degradation are operant in lung cells.

Studies with the explant system have shown that lung explants in culture will produce a collagenase which will lyse extracellular native triple helical collagen. In addition, new mechanisms of collagen degradation, probably intracellular degradation, have been defined in explants from lungs as well as from cultured lung fibroblasts. It appears that 25 to 30% of the collagen synthesized by lung explants is degraded intracellularly prior to its secretion from the cell. This phenomena is not affected by serum and is not due to proteases in the explant media secondary to artifacts of the model system. Preliminary studies with colchicine have shown that there may be an increase in intracellular degradation in the presence of colchicine. Since this drug is known to inhibit the secretion of collagen, it is possible that holding up of the secretion of the collagen molecule may allow these intracellular mechanisms to further degrade it. Studies with fibroblast cultures have demonstrated that they also rapidly degrade approximately 25% of the collagen synthesized and that these cultures can be shown to have labeled hydroxyproline within the cell suggesting again that intracellular degradation is taking place.

Studies with the collagenase assays have demonstrated that the pulmonary alveolar macrophage, human lung fibroblast, human blood monocyte, and human polymorphonuclear leukocyte all secrete a collagenase into the media when they are in culture. In the alveolar macrophage, this mechanism has been shown to be partially specific as related to the activation of collagenase. For example, in rabbits treated with BCG, their alveolar macrophages secrete more collagenase in culture than do alveolar macrophages from the normal rabbit. When the BCG aniaml macrophages are exposed to mycobacterium in vitro, they will secrete even more collagenase when alveolar macrophages from a normal animal when put under the same conditions. Most interestingly, it appears that the collagenase secreted by the alveolar macrophage is in a latent form that can be activated by a neutral protease (molecular weight 13,000) also secreted by the same cell. Thus mechanisms have been described where collagen may be degraded either intracellularly or extracellularly and control mechanisms by which these processes are regulated are being evaluated.

The control of the connective tissue of lung is fundamental to an understanding of the structure and function of this organ. In fibrotic disease and in emphysema there are apparent abnormalities of connective tissue. In fibrotic disease, the excess of collagen (at least at a local level) may be modulated by the use of mechanisms which will break down this scar tissue. In the emphysematous disorders, the destruction of connective tissue may be modulated by proteases such as collagenase present in lung. An investigation of these processes and an understanding of how they may be controlled is vital to the understanding and eventual therapy of a major source of lung disease.

Annual Report of the
Section on Molecular Pharmacology
Pulmonary Branch
National Heart and Lung Institute
July 1, 1975 to June 30, 1976

Receptors: Dysfunction of airway and vascular smooth muscle are most readily brought under pharmacological control by drugs acting at neuromoscular junctions, where signals are transmitted to receptors in muscle membrane by chemical agents. The characterization of receptors is expected to aid in the design of new drugs, in understanding the selectivity of certain drugs for receptors in specific organs and in understanding some diseases which appear to result from the formation of antibodies to receptor components or from inadequate receptor synthesis. The molecular events associated with receptor activation are being studied in this Section. Ultimately increasing emphasis will be placed on those receptors which initiate smooth muscle contraction (probably by altering calcium binding sites), since much less is known about these receptors than about the numerous mechanisms mediated by cyclic AMP. Currently, interest centers on the event which triggers all receptor activations, the adsorption of agonist on a recognition site. Research is directed to the design of techniques for receptor isolation and for studying the reactions of drugs with these large biomolecules.

Studies directed to the tagging of receptors with radioactive isotopes have shown that diazotized sulfanilic acid (DAS) in concentrations known to react irreversibly with membrane proteins do not irreversibly inactivate adrenergic or histamine receptors in rabbit aorta. DAS is thus less likely to be useful for receptor tagging than agents which react with membrane SH groups (see previous reports). A synthesis of N-(3,4-dihydroxyphenyl)-ethylmaleimide, a sulfhydryl reagent that can be labeled by coupling with microamounts of DAS, was therefore devised and carried out.

Studies of the feasibility of isolating receptors from commercially available pineal bodies have shown that some concentration (ca. 0.2 pmoles of receptor per mg of protein) can be attained in microsomal fractions and that a further 50% enrichment can be achieved with salt fractionation procedures.

Further studies of rabbit aorta have shown the presence of both H_1 (contracting) and H_2 (relaxing) histamine receptors.

Magnetic resonance and the reaction of drugs with biomolecules. Of the biophysical methods available to this Section, electron spin resonance (ESR) spectroscopy has emerged as the most useful for exploring the topography of drug and hormone binding sites. Topographic information is derived from the spectra of bound drugs into which stable free radicals (spin labels) have been introduced synthetically. Previous reports have documented that systematic application of this technique to model surfaces of increasing complexity. Spin-labeled sulfonamides, active as carbonic anhydrase (CA) inhibitors, have now been used to examine the more subtle differences in the active centers (single conical clefts) of CA isozymes. Since complete X-ray crystallographic structures of human CA I and CA II are available, these isozymes are useful models. Clefts of rabbit CA I and sheep CA II were found to be

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deeper than those of rhesus CA I and II and dog CA II. Spectral differences in the complexes of human CA I and CA II with one of the sulfonamides could be accounted for by the juxtaposition of the spin label to position 129 in the CA polypeptide chain. At this position in CA II, a phenylalanine is substituted for the leucine of CA I.

ESR studies with spin-labeled analogs of decamethonium have shown that the drug must bind to acetylcholinesterase in extended form with the two ends in different micro-environments, presumably the active site and a peripheral anionic site. Studies with the isolated nicotinic receptor of Torpedo electric tissue have yielded less topographic information but have shown that the binding of decamethonium triggers a time-dependent increase in affinity for that antagonist. The phenomenon may be related to the desensitization of nicotinic receptors.

Binding of spin-labeled derivatives of 9-aminoacridine to nucleic acid has been studied by ESR and other techniques. The data are in accord with the intercalation of the planar acridines into double-stranded helical nucleic acids. Complex spectra seen with RNA have been resolved into two components separately ascribable to single- and double-stranded nucleic acids. Acridines bearing the bulky 6-chloro and 2-methoxy substituents form complexes that are less stable, exhibit a preference for dA-dT base-pairs over dC-dG pairs, are somewhat less toxic to L1210 leukemia cells in culture and stimulate DNA-dependent RNA polymerase activity.

Other ESR studies suggest that hyperosmolality associated with freeze damage to red blood cells may be accompanied by a small decrease in the fluidity of membrane phospholipids and conformational changes in membrane proteins.

In understanding drug action, it is important to study not only the binding to nucleic acids and membrane receptors but also to serum albumin, since such binding is an important determinant of the onset, duration of action and intensity of drug effects. Quantitative studies with phenylbutazone have shown that both the number of binding sites and their affinity for the drug are reduced in albumins isolated from neonates and patients with renal failure or hepatic disease. A concentration-dependent decrease in binding sites and affinity of normal serum albumin was found to be due to association of albumin to higher molecular weight aggregates with attendant changes in conformation that could be revealed by circular dichroism.

Studies in lung: Studies on the mechanism of uptake and storage of serotonin in lungs of Wistar rats continue. The active uptake of this amine noted earlier in lung slices is not blocked by 6-hydroxydopamine or 5,6 dihydroxytryptamine, agents destructive to nerve endings, but is blocked by desmethylimipramine, tyramine, tryptamine and Lilly 110140. Since the initial rate of uptake in slices from old and young rats is identical, the increased levels of serotonin in more mature animals must be due to the appearance of intracellular storage sites. The specific activity of serotonin released from slices by reserpine after uptake of isotopically labeled amine indicates complete equiliberation of the newly taken up amine with endogenous stores. The stored isotope remains as unchanged serotonin but is rapidly converted to the

N-acetyl derivative upon release in the presence of amine oxidase inhibitors.

Studies with biogenic amines: Improved micromethods for the determination of dopamine-β-hydroxylase and of amino-acid decarboxylases have been devised. Collaborative clinical studies on the diagnostic value of histaminase and histamine assays have continued. Histaminase proved measurable in fine needle biopsy aspirates from thyroid and was found to be highly elevated in medullary carcinoid but not in other tumors. A correlation was established between plasma levels of histamine, urinary excretion of histamine and the symptoms exhibited by patients with several disorders that are presumably accompanied by histamine release. In general, urinary excretions of greater than 300 μg/24 hr are accompanied by measurable plasma histamine and high basal gastric secretion. Urinary excretion at less than this rate may be accompanied by other symptoms of histamine release, but plasma levels are not detectable and gastric secretion is normal.

Studies with anti-inflammatory drugs: As an outgrowth of earlier studies, the oral administration of aspirin and indomethacin were observed to inhibit the growth of a transplantable mouse mast-cell ascites tumor in mice and aspirin was inhibitory to a subcutaneously transplanted Lewis lung carcinoma. Further studies of this phenomenon undertaken in rat hepatoma cell cultures have shown that nonsteroidal anti-inflammatory drugs ranging in potency from meclofenamate to aspirin inhibit cell proliferation. The order of potency in inhibiting cell growth is similar to that of their anti-inflammatory activities and to the order in which they inhibit prostaglandin synthetase. The concentrations at which growth is inhibited, however, considerably exceed those necessary for synthetase inhibition. The incorporation of isotopic precursors into protein and nucleic acids of the cultured cells remains roughly proportional to the tumor mass and is not markedly affected by the addition of drugs to normal cells. The affect of drugs thus does not appear to be primarily due to the inhibition of protein and nucleic acid synthesis. Salicylates, however, are anomalous in that they stimulate these synthetic reactions in small concentrations. The mechanism of this inhibition may be relevant to the anti-inflammatory properties of drugs.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02401-04 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Models of Lung Growth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	A.L. Horwitz	Research Associate	PB NHLI

COOPERATING UNITS (if any)

William M. Thurlbeck, M.D., Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada.

LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.9	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

Two models of lung growth are being used to evaluate the control of connective tissue accumulation as related to normal alveolar structure. In normal neonatal lung growth in the rabbit, glycosaminoglycan content, types and synthesis have been quantitated. The changes seen with lung growth may have significant influence on the mechanical properties of lung during these periods. In post-pneumonectomy lung growth in the rabbit, the total number of alveoli double and alveolar structure appears normal. This data, together with prior information on the control of collagen synthesis in this model, suggest there are complex controls which may be harnessed to induce alveolar regeneration.

Project Description:

Objectives: The major function of the lung is to transfer oxygen from the atmosphere to the blood and carbon dioxide from the blood to the atmosphere. This function is provided by the unique structure of lung and its mechanical properties in relation to changes in intrapleural pressure. The stucture and mechanical properties of lung are intimately dependent on the connective tissue composition of this organ. The presence of these connective tissue components is controlled by a balance of synthesis and degradation. Two models are being used to investigate the control of connective tissue synthesis and degradation during lung growth: (1) changes during normal neonatal lung growth; and (2) "compensatory" lung growth following unilateral pneumonectomy.

Prior studies have defined the normal amounts of collagen and rates of collagen synthesis in the lung in both models. The molecular basis of the controls in collagen synthesis have been partially defined using cell-free systems to quantitate collagen messenger RNA bearing polysomes during neonatal lung growth. (See projects # Z01 HL 02401-03 PB, and # Z01 HL 02402-03 PB). Preliminary studies have shown changes in glycosaminoglycan quantities, types and synthesis during neonatal lung growth. In the past year, the changes in glycosaminoglycans during neonatal lung growth have been more carefully defined and the morphological correlates of post-pneumonectomy lung growth have been evaluated.

Methods: Glycosaminoglycan composition and synthesis are quantitated in lungs of different age rabbits. The lungs are removed, homogenzied, and glycosaminoglycans are isolated following protein digestion. Total glycosaminoglycan content is determined by measurement of uronic acid or hexosamine. The identification and quantitation of glycosaminoglycan types in lung were done by a combination of column methodologies along with specific enzymes. The rate of total glycosaminoglycan synthesis by lung explants from different age rabbits were done using [1-^{14}C]glucosamine as a precursor of the glycosaminoglycans. Total glycosaminoglycan synthesis was quantitated at different time intervals and the rate of synthesis of glycosaminoglycans was assayed using methods described above and by relating synthesis to DNA and specific activity of the glycosaminoglycan precursors in the tissue. The specific types of glycosaminoglycans synthesized by the explants were determined using column methodologies along with enzymatic digestion of the labeled glycosaminoglycans.

The right lung from rabbits that had undergone pneumonectomy one month previously were compared to the right lung of sham operated controls. The lungs were removed from the animal, inflated to 15 cm water pressure and fixed with glutaraldehyde. Using classic methodologies of lung morphometry, we have quantitated the number of alveoli, number of alveolar ducts, percentage of lung that is air, percentage of lung that is tissue, percentage of lung tissue that is parenchyma and percentage of lung tissue that is alveolar duct. These studies were done in a blind fashion with those doing the morphometry having no knowledge of the control or

experimental animals.

Major Fingings: Parenchymal glycosaminglycan content ranges from 0.2 to 0.4 percent (wt/dry wt), with highest concentration in adult lung. There are significant changes in types of glycosaminoglycans present at different ages. Fetal lungs contain a relatively high proportion of chondroitin 4-sulfate, while the glycosaminoglycans in lung parenchyma of older animals was predominantly dermatan sulfate, heparan sulfate and heparin. The rate of synthesis of total glycosaminoglycans per cell increased with development to a maximum in lung from weanling rabbits and fell to low rates of synthesis in mature rabbits. Fetal rabbit lung parenchyma synthesized mostly hyaluronic acid and heparan sulfate, while in weanling rabbit parenchyma, hyaluronic acid and chondroitin 4/6-sulfate synthesis was greatest. In mature animals, the rates of synthesis of all types of glycosaminoglycans were relatively low but there was a relatively greater emphasis on synthesis of dermatan sulfate and heparin.

Preliminary morphometric data of post-pneumonectomy lung growth suggest that one month after pneumonectomy, the remaining lung doubles in size with this doubling being associated with a doubling of the number of alveoli. There appears to be no abnormalities in alveolar ducts, parenchymal tissue thickness, or other parameters of alveolar size. Thus it appears that post-pneumonectomy lung growth in the rabbit is remarkably similar to normal neonatal lung growth in the same animal.

Significance to Biomedical Research and Institute Program: These two models provide a means to study the lung during periods of "turning off" or "turning on" of connective tissue synthesis and degradation. With a comprehensive approach at the cellular and sub-cellular levels, it should be possible to understand the control of structural and non-structural protein synthesis in lung. This has obvious applications to control of fibrosis in the interstitial lung disorders and to the replacement of alveoli in the emphysematous disorders.

Proposed Course to Project: Other methods developed in the laboratory to evaluate the control of connective tissue during lung growth are being applied to these two models. Of particular interest are the quantitation of collagenase induced degradation of collagen in the extracellular space and intracellular degradation of collagen in the interstitium (see project report # Z01 02409-01 PB). These investigations are to be expanded into attempts at manipulation of lung collagen degradation with proteolytic inhibitors and pharmacologic agents . Studies are to be performed on the continued evaluation of the factors responsible for the growth of the lung following unilateral pneumonectomy. For example, serum growth factors may play important roles. These types of studies will be combined with methods that are being developed to evaluate cultured lung cells (see project report # Z01 HL 02403-03 PB). There will be continued investigation of the sub-cellular mechanisms involved in control of the synthesis of connective tissue complements during lung growth. This will involve utilizing cell-free systems to isolate collagen messenger RNA for specific collagen

types, to prepare labeled DNA probes from purified collagen messenger RNA, and to use the DNA probes to quantitate gene products in the control of collagen synthesis. In combination with projects of cultured lung cells these models will be evaluated on a sub-cellular level by investigating the interactions of connective tissue components between themselves and lung cells and the effect of connective tissue components in controlling the differentiated properties of lung cells. These normal models are being evaluated in terms of the role of inflammatory and immunologic mechanisms in the maintenance of normal lung connective tissue.

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July 1, 1976 to June 30, 1976

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Isolation, Culture and Characterization of Lung Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	A.J. Hance	Clinical Associate	PB NHLI
	A.L. Horwitz	Research Associate	PB NHLI
	N.A. Elson	Staff Investigator	PB NHLI
	K. Bradley	Chemist	PB NHLI
OTHER:	S. McConnell-Breul	Pharmacologist	PB NHLI
	J.A. McDonald	Research Associate	PB NHLI
	R.S. Bienkowski	Staff Fellow	PB NHLI
	J.B. Karlinsky	Staff Fellow	PB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.7	3.2	1.5

SUMMARY OF WORK (200 words or less - underline keywords)

The complexity of the lung necessitates simplification before biochemical methods can be fully utilized to understand the maintenance of lung structure and function in health and disease. One approach to this problem is by utilizing cell culture methods to isolate and maintain homogeneous populations of lung cells. These methods are being used to evaluate lung collagen synthesis, the maintenance of the differentiated state in culture, glycosaminoglycan synthesis, biochemical identification of cells in culture, mechanisms of collagen degradation, hereditary disorders of connective tissue, composition of lung connective tissue, maintenance of lung cells from patients with pulmonary fibrosis, immunologic interactions in fibrotic disease and in vitro models of environmental lung injury.

933

PHS-6040
(12-75)

Project Description:

Objectives: The use of biochemical technology to understand human lung disease is limited by the complexity of the organ, the unavailability of large quantities of lung cells, and the multitude of variables involved in attempting to study the lung in situ. Even though the biochemist can approach the study of lung cells with a vast armamentarium of concepts and sophisticated methodology, the inherent complexity of dealing with multiple cell types immediately hinders the sorting out of the contribution of each cell type to the biochemical parameters being measured. A lung parenchyma devoid of blood elements and cells of the alveolar lumen (a very difficult state to achieve experimentally) still has four major cell types (endothelial cell, mesenchymal cell, alveolar type I cell and the alveolar type II cell). For example, attempts to quantitate dipalmitoylphosphotidylcholine, in an alveolar type II cell in such a parenchyma would, by definition, necessitate large quantities of dipalmitoylphosphotidylcholine, since the type II cell represents less than 10 percent of the cells in the parenchyma. In addition to the problems of cell purity, the study of lung cells in situ is compounded by the multitudes of factors which impinge on each cell type to attempt to vary its inherent properties. Thus, to reduce the number of (mostly unknown) variables to a minimum, it has become necessary to develop techniques by which individual populations of lung cells can be isolated, cultured and studied. One of the primary objectives of this laboratory is to develop the technology to culture the cells from the lung that are important in the maintenance of normal parenchymal structure in health and disease. Of particular interest are the processes by which these cells synthesize and degrade the extracellular connective tissue matrix of the alveolus.

Methods: In general there are three methods used for the isolation and culture of lung cells. The easiest is by direct isolation, but this is limited to one cell type, the alveolar macrophage. This cell is the most readily available cell in the lung parenchyma. By broncho-alveolar lavage methods, $30\text{-}100 \times 10^6$ macrophages can be obtained per adult rabbit. Broncho-alveolar lavage in the human can yield $10\text{-}15 \times 10^6$ cells from the normal. These macrophages can be maintained in culture for 7 to 30 days. In culture, the macrophages adhere to surfaces, phagocytize, kill bacteria and release a wide variety of enzymes.

The second method used to isolate lung cells is by capitalizing on specific cell physicial and/or biological properties. Implicit in the definition of the "differentiated" cell is the concept that each cell population has different properties. Differences in density and/or adherence to surfaces are the most common parameters exploited in cell isolation procedures. The methods used to group parenchymal cells into individual populations are just being refined. Before these cells can be grouped into individual populations, they must first be separated by enzymatic methods. Once free from the alveolar septum, the alveolar type II cell can be substantially purified by allowing contaminating macrophages to phagocytize heavy materials which allow them to be removed from type II cells by sedimentation methods. The

resulting type II cells can then be analyzed and/or cultured.

The third method of isolation of specific lung cells are by culturing methods. Basically there are two variations of these methods. In the "hardiest will survive" method, the lung parenchyma is dispersed with enzymes and the resulting cells are allowed to grow in primary culture. If special methods are not used, one cell type will usually "overgrow" the plate. When confluency is reached, the cells are replated (passaged) and the hardiest cell population, usually a mesenchymal cell, obtains supremacy and eventual homogeneity. In the past year we have used the methods to isolate and characterize two lung mesenchymal (fibroblast) populations; NB-6 from newborn rabbit parenchyma and HFL-1 from a 16-week-old human fetal lung. In the second variation of these methods, the cloning techniques are used after lung cells are enzymatically dispersed from parenchyma and plated at very low cell densities. Populations of cells growing from one cell are recovered and allowed to grow to homogeneity. AK-D, a fetal cat "epithelial" cell, is an example of this method. This cell may be a tissue culture analog of the alveolar type I cell. We have also utilized this method to grow fetal cat fibroblast cells to more than 20 population doublings.

Although the advantage of studying specific cell types in culture is apparent, there are major problems to be considered when using this technology to study the biochemical processes which maintain lung structure and function. Using electron microscopic methods, it is relatively easy to identify the major cell types of the lung when these cells are in situ. However, when these cells are in culture, many morphological features are lost; particularly the feature of anatomic location. To attempt the problem of identification of lung cells in culture, we have utilized a method of displaying newly synthesized, labeled, secreted proteins on molecular weight "maps." These maps can then be compared from different cultures, cultures at different time in passage, or cultures in different species, cultures in different organs and cultures of cells of varying age.

Major Findings: The culture techniques described above have been utilized to isolate and/or culture the following cell types: (1) alveolar macrophages from the adult human and adult rabbit; (2) fibroblasts from the adult human lung, fetal human lung, newborn rabbit lung, newborn rat lung, fetal cat lung, and adult rabbit skin; (3) endothelial cells from the human umbilical vein, smooth muscle cells from the human umbilical vein; (4) epithelial cells from the fetal cat lung; and (5) alveolar type II cells from the adult rabbit lung. These cells have been utilized in the following studies:

(1) Methods have been developed to quantitate the absolute rate of collagen synthesis and degradation in cell cultures. In addition, cyanogen bromide methods have been used in conjunction with carboxymethyl cellulose chromatography, sodium dodecyl sulfate acrylamide gels, and isoelectric focusing gels to quantitate the relative amounts of Type I and Type III collagen synthesized by cells in culture (see Project # Z01 HI 02406-03 PB).

(2) Using molecular weight maps it has been determined that the human fetal lung fibroblast secretes the same proteins at passage 3 as passage 15. The rates of collagen synthesis per cell are identical as are the rates of total non-collagen protein synthesis throughout this time period.

(3) Methods used to quantitate the types of glycosaminoglycans secreted by cells are being utilized to compare the differentiated properties of the alveolar epithelial cells with alveolar mesenchymal cell. (See Project # Z01 HL 02401-04 PB).

(4) The methods of molecular weight maps are being utilized to characterize different types of lung cells. The patterns of secreted proteins of the alveolar type II cell, blood monocyte, alveolar macrophage and fibroblasts are completely different. These maps are characteristic of these cell types and can be used in addition to morphology to specify the types of cells in culture.

(5) Evaluation of the collagen synthesized by lung cells in culture shows that significant proportion of the collagen synthesized is degraded, probably intracellulary. Methods have been developed to quantitate this process and evaluation is ongoing to attempt to manipulate intracellular collagen degradation. Culture methods are also being used to evaluate the synthesis, secretion and mechanisms of action of collagenase secreted by the alveolar macrophage and fibroblasts in culture (see Project # Z01 HL 02409-01 PB).

(6) Skin fibroblasts are being utilized to evaluate, diagnose and define the hereditary disorders of connective tissue (see Project # Z01 HL 02408-01 PB). This data is being combined with information of connective tissue in lung parenchyma and with sophisticated studies of pulmonary function abnormalities in these disorders. By this means an understanding of biochemical-mechanical correlates of lung function are being achieved (see Project # Z01 HL 02407-02 PB).

(7) Studies with cultured cells have suggested that the glycosylation of collagen synthesized in culture is markedly different from that of the characteristic collagens found in these organs. This implies either there are: (a) significant post-translational mechanisms which modulate collagen carbohydrates after their synthesis; or (b) that new, as yet undescribed, collagens are being synthesized by these cells in culture (see Project # Z01 HL 20408-01 PB and Project # Z01 HL 02406-03 PB).

(8) Cell cultures from patients with fibrotic lung disease and normal humans are being utilized in cell-cell interaction studies involving immune competent components from patients with fibrotic lung disease (see Project # Z01 HL 02407-02 PB). For example, lymphocytes from patients with pulmonary fibrosis are placed on fibroblasts labeled with ^{51}Cr to demonstrate that these lymphocytes induce lysis of the fibroblasts. These cell-cell models are being further investigated to evaluate pathogenic mechanisms of the inflammatory and immune systems in fibrotic disease states.

(9) In the fibrotic lung disorders the patient is usually biopsied at one time in the clinical course. These biopsies are being utilized to culture lung cells from these patients. Besides the studies mentioned above, the establishment of diploid cell lines of the cells responsible for collagen synthesis in these disease states allow investigations at the molecular level. These cells are being utilized for preliminary studies and are being frozen away for recall as the methods develop. In this way, the genetic information of the patients with these disorders can be saved for long periods of time.

(10) Cultured lung cells are being utilized in cell-cell interaction studies for models of pulmonary fibrosis. For example, pulmonary alveolar macrophages are being fed silica or other environmental pollutants and the supernatant from these cultures are being evaluated for the effect on the differentiated state of lung cells including rates of collagen synthesis and degradation.

Significance to Biomedical Research and Institute Program: The mechanisms by which the differentiated state is controlled in the lung are the mechanisms by which the lung maintains its structure and function. In the fibrotic lung disorders, for example, these mechanisms are presumably derranged so that collagen is synthesized in an abnormal fashion. The establishment of diploid cell lines of the cells responsible for the maintenance of alveolar structure and function in health and disease allows investigations at the molecular level. This includes identification of the normal mechanisms of maintenance of lung and how these mechanisms are varied following lung injury.

Proposed Course of Project: There will be continued establishment of different cell lines, the investigation of the various proteins and other macromolecules made by these cells, and inquiry into the mechanisms of the maintenance of differentiated states. One area of particular interest is the control of synthesis and degradation of connective tissue either because of the inherent properties of lung cells or by cell-cell interactive mechanisms. As these techniques are established in normal animal lung cells, they are applied to the study of normal human cells and to the study of human lung cells from biopsy specimens of patients with lung disease.

Publications:

Hance, A.J., Bradley, K. and Crystal, R.G.: Lung collagen heterogeneity. Synthesis of type I and type III collagen by rabbit and human lung cells in culture. J. Clin. Invest. 57: 102-111, 1976

Hance, A.J., Horwitz, A.L., Cowan, M.J., Elson, N.A., Collins, J.F., Bienkowski, R.S., Bradley, K.H., McConnell-Breul, S., Wagner, W.M. and Crystal, R.G. Biochemical approaches to the investigation of fibrotic lung disease. Chest 69 (Suppl.): 257-261, 1976

Crystal, R.G.: Biochemical Processes in the Normal Lung. In Bouhuys, A. (Ed.): Lung Cells in Disease. Amsterdam, North Holland-ASP Biological and Medical Press, 1976 (in press)

938

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02404-04 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Accumulation of Collagen in Human Lung in Health and Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	J.D. Fulmer	Staff Investigator	PB NHLI
	R.S. Bienkowski	Staff Fellow	PB NHLI
OTHER:	K.H. Bradley	Chemist	PB NHLI
	A.J. Hance	Clinical Associate	PB NHLI
	S. McConnell-Breul	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

Fibrotic lung disease is associated with scarring of the alveolar interstitium. Studies with surgical biopsy specimens and post-mortem lung have revealed that in idiopathic pulmonary fibrosis and in pulmonary fibrosis associated with lung tumors, the concentration of lung collagen and the rate of synthesis of lung collagen is actually normal. These findings suggest the classic concepts of pulmonary fibrosis need readjustment and that the lung fibrotic states may be associated with changes in types of collagen or redistribution of collagen rather than simply increased amounts.

939

PHS-6040
(12-75)

Project Description:

Objectives: The connective tissue of the lung is fundamental for lung structure and mechanical properties. In addition, the interstitial lung diseases, many of which result in pulmonary fibrosis, represent approximately 20% of lung disorders (other than infectious diseases). Almost nothing is known about the composition, synthesis or degradation of human lung collagen. With the ultimate aim being an ability to control pulmonary fibrosis through molecular mechanisms, our laboratory is developing the technology to quantitate human lung collagen composition, synthesis and degradation.

Methods: Lung tissue is obtained from open lung biopsy or post-mortem specimens from patients with pulmonary fibrosis or from normal areas of lobes removed for localized lung tumors. In the biopsy specimens, rates of collagen synthesis are quantitated. In the biopsy and post-mortem specimens, collagen concentrations are quantitated as are the relative concentrations of the different types of collagen.

Major Findings: Prior studies have established the values for the concentration of collagen and rates of collagen synthesis in control lungs. Studies with 8 patients with idiopathic pulmonary fibrosis and 4 patients with pulmonary fibrosis secondary to tumors (compared to 6 controls) have shown: (1) the concentration of collagen (amount per unit dry weight) in all 3 groups are identical; and (2) the rate of collagen synthesis per cell (averaged over all cells in the biopsy) are identical.

Quantitation of multiple (40-50/lung) biopsies of post-mortem specimens of lungs of patients with idiopathic pulmonary fibrosis compared with normal (death due to non-lung causes) controls have demonstrated that the lack of differences between the collagen concentration in idiopathic pulmonary fibrosis vs. controls is not due to a "sampling problem" since the distribution of values obtained from the post-mortem lungs was the same in both groups.

Significance to Biomedical Research and Institute Program: The process of normal gas exchange is critically dependent on the form and function of each of the components comprising the lung parenchyma. Collagen, the most abundant protein in this structure, provides structural integrity and, most likely, functions that are fundamental to lung mechanical properties. Idiopathic pulmonary fibrosis is a disease state associated with the presence of "scar" in the alveolar interstitium. In biochemical terms, "scar" or "fibrosis," means collagen deposition in excess amounts, abnormal type and/or abnormal location. Through the techniques developed in our laboratory, it has been possible to: (1) quantitate the concentration of collagen in biopsy specimens of human lung; and (2) quantitate the rate of collagen synthesis (averaged over all lung cells) in lung biopsies. The surprising results of these studies strongly suggest: (1) there is no increase in collagen concentration in idiopathic pulmonary fibrosis; and (2) there is no difference in the rates of collagen synthesis in idiopathic pulmonary fibrosis compared to normal. Interestingly, patients who have

pulmonary fibrosis secondary to tumors in their lungs have similar findings. In comparison, rabbits who have experimentally induced radiation fibrosis have significant elevations of collagen concentration preceded by elevations in rates of collagen synthesis. Thus, we have to rethink the biochemical concepts of the "fibrotic" disease state in the human lung. Idiopathic pulmonary fibrosis is not simply a disease in which the lung makes more collagen, but is probably a much more complex process. For example, IPF may be associated with: (1) a relative increase in the population of cells that synthesize collagen; (2) a shift in the types of collagen synthesized; (3) a decrease in the rate of collagen proteolysis; or (4) a localized derrangement where collagen is synthesized or laid down. From a technological point of view, the last possibility will be the most difficult to quantitate.

These concepts also have application to the emphysematous disorders where there is a destruction of the connective tissue comprising the lung with either absent or ineffective remodeling of the connective tissue. Of particular interest is the pathogenesis of the α1-antitrypsin group of patients, who have an inherited disorder of the serum α1 globulins associated with emphysema. The techniques described can be used to directly test the influence of serum proteins on protecting the lung from proteolysis.

Proposed Course of Project: Investigations are ongoing to evaluate the possibility that: (1) local accumulation of collagen in the fibrotic disorders is secondary to decreased degradation of collagen rather increased synthesis; (2) that there is a change in the types of collagen present in the parenchyma, yet the total amount of collagen is the same (see Project # Z01 HL 0205-03 PB); and (3) that the disease is associated with a restructuring of collagen in the parenchyma leaving the same amounts but in abnormal locations.

Once the pathological abnormalities are understood at the biochemical level, it should be possible to predict how therapy may be applied to enable the lung to restructure itself so that it may function normally.

Publications:

Fulmer, J.D., Roberts, W.C. and Crystal, R.G.: Diffuse fibrotic lung disease: a correlative study. Chest 69 (Suppl.): 263-265, 1976

Fulmer, J.D. and Crystal, R.G.: The Biochemical Basis of Pulmonary Function. In Crystal, R.G. (Ed.): The Biochemical Basis of Pulmonary Function. New York, Marcel Dekker, 1976, pp. 419-466

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02405-03 PB

PERIOD COVERED

July 1, 1976 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Experimental Models of Fibrotic Lung Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	A.J. Hance	Clinical Associate	PB NHLI
	W.M. Wagner	Guest Worker	PB NHLI
OTHER:	J.D. Fulmer	Staff Investigator	PB NHLI
	N.A. Elson	Staff Investigator	PB NHLI
	S. McConnell-Breul	Pharmacologist	PB NHLI
	J.B. Karlinsky	Staff Fellow	PB NHLI
	S.E. Weinberger	Clinical Associate	PB NHLI
	J. Moss	Research Associate	CM NHLI

COOPERATING UNITS (if any)

Laboratory of Cellular Metabolism, NHLI

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.5	0.6

SUMMARY OF WORK (200 words or less - underline keywords)

Animal and cell culture models of pulmonary fibrosis are being developed. X-radiation of the one lung in the rabbit (3000 to 6000 R) results in increases in the rates of collagen synthesis per cell and accumulation of collagen. Methods have been developed to quantitate the rates of collagen synthesis in lung cells in culture and to quantitate the relative amounts of types of collagen being synthesized. These techniques are being combined with in vitro analogs of tissue injury to evaluate the modulation of inflammatory and immune mediators on the control of collagen accumulation in the lung.

942

PHS-6040
(12-75)

Project Description:

Objectives: A major goal of this laboratory is to understand and eventually control the mechanisms involved in connective tissue synthesis and degradation in human lung in diseases involving these components. Because the availability of human lung biopsy material is limited, it necessitates that any biochemical techniques applied to this tissue is worked out first in model systems, thus insuring the maximum amount of information and the maximum benefit to the patient. For that reason two groups of models of fibrotic disease are being developed and evaluated: (1) animal models; and (2) tissue culture models.

Methods: (1) Animal models: Three models are being evaluated. (a) X-radiation to the lung of rabbits. (b) Administration of paraquat to Syrian hamsters. (c) Administration of bleomycin to mice. In the radiation model, 3000 R or 6000 R are administered to the right lung of a rabbit. In the right irradiated lung, left non-irradiated lung and control lungs of litter mate non-irradiated animals, the collagen content, rates of collagen synthesis and rates of non-collagen protein synthesis are quantitated and compared to light and electron-microscopic sections of fixed, inflated lungs from these animals. The paraquat and bleomycin models are still being evaluated. Preliminary studies have suggested the optimal dose for paraquat is 15 mg/kg (one dose) and the optimal dose for bleomycin is 10 mg/kg daily for 1 week.

(2) Tissue culture models: The development of methods to culture and continually passage lung cells in culture has enabled us to begin the development of *in vitro* models of lung injury and to quantitate the cellular response to lung injury. Several approaches are being used.

(a) Methods have been developed to quantitate the rates of collagen synthesis per cell in cultured lung cells. These measurements are based on the incorporation of [^{14}C]proline into [^{14}C]hydroxyproline, the release of [^{14}C]proline in newly synthesized collagen by Clostridial collagenase, the specific activity of [^{14}C]proline within the cells and cell number.

(b) Methods have been developed to quantitate the relative amounts of Type I and Type III collagen synthesized by cultured lung cells (see Project # Z01 HL 02406-03 PB).

(c) These methods are being combined with methods to parallel inflammatory and/or immune injury to lung cells. For example, cultured alveolar macrophages derived from BCG-treated animals are exposed to mycobacterium *in vitro*. The supernatant of these cultures are then added to lung fibroblasts in culture. Another method used is to take lymphocytes from patients with pulmonary fibrosis (these patients' lymphocytes are sentizied to collagen; see Project # Z01 HL 02407-02 PB), expose these lymphocytes to collagen *in vitro*, take the supernatant and add this to lung fibroblasts in culture.

Using the methods in (a) and (b), the effect of these stimuli on rates and types of collagen synthesized by lung cells can be determined.

Major Findings: (1) Animal models: The right lung of the irradiated animals (both 3000 and 6000 R) show significant increases in collagen content 8 to 10 weeks after irradiation. This collagen accumulation is paralleled by increases in the rates of collagen synthesis per cell. Morphologic studies show cellular injury (particularly endothelial cell) prior to the increase in rates of collagen synthesis or accumulation of collagen. These findings are significantly different from similar quantitative measurements in biopsy specimens from patients with idiopathic pulmonary fibrosis (see Project # Z01 HL 02407-02 PB).

(2) Tissue culture models: In fibroblasts from rabbit and human lungs, approximately 4 to 5% of the total proteins synthesized are collagen. This finding is invarient during the life of the cell (e.g., passage 5 compared to passage 15) as long as the states of cell density (e.g., log vs. confluent phase of growth) and intracellular pools of amino acids are considered. Preliminary studies have suggested that comparing the confluent phase of culture to the log phase of culture may be a model system in which to study changes in the control mechanisms governing the types of collagen synthesized by lung cells. The modulation of these controls by inflammatory and immune mechanisms is still in the developmental stage.

Significance to Biomedical Research and Institute Program: As we learn to define the animal and tissue culture models of fibrotic lung disease, we should be able to understand the sensitive control points where we can interrupt the fibrotic process with pharmacologic agents.

Proposed Course to Project: Continued development of these models with particular emphasis on the quantitative description of the fibrotic process including the type of collagen synthesized and how this may be influenced with drugs. Other projects in our laboratory such as cell-free collagen synthesis will be applied to these models as the technology develops.

Publications:

Hance, A.J., Horwitz, A.L., Cowan, M.J., Elson, N.A., Collins, J.F., Bienkowski, R.S., Bradley, K.H., McConnell-Breul, S., Wagner, W.M. and Crystal, R.G.: Biochemical approaches to the investigation of fibrotic lung disease. Chest 69 (Suppl.): 257-261, 1976

Fulmer, J.D. and Crystal, R.G.: The Biochemical Basis of Pulmonary Function. In Crystal, R.G. (Ed.): The Biochemical Basis of Pulmonary Function. New York, Marcel Dekker, 1976, pp. 419-466

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02406-03 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Heterogeneity of Lung Collagen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	A.J. Hance	Clinical Associate	PB NHLI
OTHER:	K.H. Bradley	Chemist	PB NHLI
	R.S. Bienkowski	Staff Fellow	PB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.9	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

The lung is composed of at least four collagen types. Type I [$\alpha 1(I)_2 \alpha 2$] is the fibrillar collagen found in the parenchyma and blood vessels. Type II ($[\alpha 1(II)]_3$) is cartilage collagen of the tracheo-bronchial tree. Type III ($[\alpha 1(III)]_3$) is probably reticulin found in the parenchyma and around blood vessels. Type IV ($[\alpha 1(IV)]_3$) is probably basement membrane collagen, but it has not as yet been fully characterized. Methods are being developed to quantitate Types I and III collagen in lung parenchyma and cell culture in order to help understand the control of lung collagen in health and disease.

945

PHS-6040
(12-75)

Project Description:

Objectives: Collagen is the most abundant protein in lung comprising 10-20% of the adult lung by dry weight. There are more than 40 cell types in lung including mesenchymal cells, epithelial cells, endothelial cells and smooth muscle cells, all of which have been implicated to synthesize collagen. The primary objective of this project is to identify the heterogeneous types of collagen in lung and the cells that synthesize it.

Methods: The primary method for evaluating types of collagen and quantitating the amounts present is by using cyanogen bromide peptide mapping techniques. This involves a combination carboxymethyl cellulose chromatography, sodium dodecyl sulfate acrylamide gels and isoelectric focusing gels. Because there are 5 to 10 methionine residues per 1000 amino acid residues in collagen α chains, cyanogen bromide is particularly suitable for cleaving collagen (it cleaves only at methionine residues) leaving peptides which are few enough in number to be able to handle. Although collagen is very difficult to extract from intact lung, combinations of salt extraction, acetic acid extraction, guanidine extraction plus the use of enzymes (e.g., pepsin) to release collagen chains from the connective tissue matrix have enabled the evaluation of types of collagen present in the lung parenchyma.

Methods have also been developed using a similar technology to quantitate and identify the types of collagen synthesized by explants of the lung maintained in vitro and by tissue culture systems (described in Project # Z01 HL 20403-03 PB).

Major Findings: The lung parenchyma is composed of at least Type I collagen (two α1(I) chains and one α2 chain in the form $[\alpha 1(I)_2 \alpha 2]$) and Type III collagen (three α1(III) chains in the form $[\alpha 1(III)]_3$). Because lung has the morphological evidence of extensive basement membrane (70 m^2 in the adult human lung), it is likely that basement membrane collagen is present in the lung parenchyma as well. However, studies quantitating the 4-hydroxyproline to 3-hydroxyproline in the parenchyma of adult human lung have revealed ratios of approximately 100. Therefore, if basement membrane in lung is similar to basement membrane from other organs (kidney basement membrane has very large quantities of 3-hydroxyproline compared to 4-hydroxyproline), then less than 1% of the lung parenchyma is basement membrane. It is also possible that lung basement membrane is different than kidney basement membrane in which new markers will have to be developed for its evaluation and quantitation.

The tracheo-bronchial tree contains Type II collagen (cartilage collagen) and the major blood vessels contain at least Type I collagen and probably Type III collagen as well.

It is now possible to quantitate the types of collagen synthesized by lung cells in culture, and methods are being evaluated to quantitate types of collagen synthesized by lung explants in vitro. With these methods, it

should be possible to soon quantitate the relative type of different collagens being synthesized in the lung parenchyma in health and disease. In the tissue culture systems, these methods are adaptable to understanding the molecular control of the synthesis of these collagen types and how this control might be modulated when these systems go awry in lung disorders.

Significance to Biomedical Research and Institute Program: Normal lung structure and function depends on the collagen comprising it. During the developmental process there are major changes in the control of collagen synthesis. An understanding of these mechanisms will help toward an understanding of the pathological processes in diseased lung.

The fibrotic lung diseases have many primary stimuli including inhalation of of toxic materials, hypersensitivity states and radiation injury. In addition, pulmonary fibrosis is often associated with systemic diseases such as scleroderma. It is likely that these disorders are associated with injury to different lung cells, suggesting the possibility that there may be a heterogeneity in the types of collagen synthesized in these different lung diseases. It may be possible to classify the interstitial lung disorders on the basis of types of collagen synthesized in a similar fashion to the lipoprotein disorders and certain hemoglobin disorders.

The heterogeneity of lung collagen may also be a crucially important determinant of lung mechanical properties in the normal development of lung.

Proposed Course of Project: The next major areas are to finish developing methods to quantitate Types I and III collagen in the parenchyma. Basement membrane collagen (Type IV collagen) will have to be evaluated, and methods developed to understand its synthesis and degradation. To do this it is necessary to isolate and purify this basement membrane collagen of which several methods have become available in recent months. Once that is accomplished, it should be possible to quantitate the rates of synthesis, degradation and amount of each collagen type in normal lung development and in human lung disease.

Publications:

Hance, A.J. and Crystal, R.G.: The connective tissue of lung. Amer. Rev. Resp. Dis. 112: 657-711, 1976

Hance, A.J., Bradley, K. and Crystal, R.G.: Lung collagen heterogeneity. Synthesis of type I and type III collagen by rabbit and human lung cells in culture. J. Clin. Invest. 57: 102-111, 1976.

Hance, A.J. and Crystal, R.G.: Collagen. In Crystal, R.G. (Ed.): The Biochemical Basis of Pulmonary Function. New York, Marcel Dekker, 1976, pp. 215-271.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02407-02 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of Patients with Fibrotic Lung Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	J.D. Fulmer	Staff Investigator	PB NHLI
OTHER:	K.H. Bradley	Chemist	PB NHLI
	N.A. Elson	Staff Investigator	PB NHLI
	R.S. Bienkowski	Staff Fellow	PB NHLI
	A.J. Hance	Clinical Associate	PB NHLI
	S.E. Weinberger	Clinical Associate	PB NHLI
	A.L. Horwitz	Research Associate	PB NHLI

COOPERATING UNITS (if any)

See attachment.

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.7	2.4	2.3

SUMMARY OF WORK (200 words or less - underline keywords)

Idiopathic pulmonary fibrosis is a chronic, fatal lung disorder grouped with the fibrotic lung diseases. Studies with these patients have correlated physiologic parameters with histopathology; quantitated gas exchange abnormalities; characterized content and rates of synthesis of collagen in lung biopsies; demonstrated elevated inflammatory cells and immunoglobulin-G in broncho-alveolar lavage fluid, developed methods to quantitate the uptake of ^{67}Gallium-citrate in lung as a non-invasive measure of lung inflammation; and shown that peripheral blood lymphocytes from these patients recognize collagen as antigenic and produce lymphokines when exposed to it in vitro. Evaluation of drug therapy of this disease with corticosteroids and immunosuppressive agents is still ongoing.

Cooperating Units:

J. Moss, Laboratory of Cellular Metabolism, NHLI
J. Oppenheim, Laboratory of Microbiology and Immunology, NIDR
T. Kravis, Scripps Clinic and Research Foundation, La Jolla, California
A. Ahmed, Naval Medical Research Institute, Bethesda, Maryland 20014
K. Mittal, Bureau of Biologics, FDA
B. Line, Division of Nuclear Medicine, CC
H. Reynolds, Section of Bacterial Diseases, LCI, NIAID
F. Wenzel, Marshfield Clinic, Marshfield, Wisconsin
W. Roberts, Section on Pathology, IR, OD, NHLI
V. Ferrans, Section on Pathology, IR, OD, NHLI
R. Dolin, Laboratory of Clinical Investigation, NIAID

Project Description:

Objectives: The fibrotic lung disorders represent 15-20% of the non-infectious disorders of the lung. There are more than 130 separable disease entities associated with pulmonary fibrosis, but they can be categorized into ten general groups (occupational and environmental etiologies, drug reactions, physical agents, infectious disorders, secondary to cardiac disease, pulmonary vascular disease, neoplastic disease, congenital and familial, unknown etiology but with characteristic morphology, idiopathic pulmonary fibrosis). The Pulmonary Branch, NHLI, has undertaken a detailed study of patients with pulmonary fibrosis, particularly those with environmental etiologies and those with idiopathic pulmonary fibrosis (IPF). The latter represents a chronic, devastating illness, invariably resulting in death an average of 4 to 5 years from the onset of symptoms. Although approximately 5-10% of patients with IPF respond to corticosteroids, there is no known treatment for the remainder. The objectives of our studies are as follows:

(1) To determine the etiology of IPF.
(2) To follow patients with IPF longitudinally to determine (a) the natural history of the disorder and (b) which pulmonary function parameters are most sensitive to the disease process.
(3) To correlate pulmonary alveolar constituents (alveolar lavage fluid and cells) in IPF with histopathology and pulmonary function parameters as well as with alveolar constituents in other fibrotic disorders.
(4) To correlate radioisotopic monitors of lung function (ventilation, perfusion and gallium scans with the disease process.
(5) To correlate the histopathology in IPF with biochemical and functional parameters.
(6) To study the therapy of IPF.
(7) To study abnormal inflammatory and immunologic processes associated with the continued pathogenesis of the IPF.
(8) To study genetic determinants of IPF.

Methods: Patients admitted to the Pulmonary Branch Clinical Service enter an extensive protocol which includes: detailed medical and pulmonary history and physical exam, routine serologic, roentgenographic and EKG studies, serologic studies aimed at immune processes; pulmonary function studies including lung volumes, flow rates, diffusing capacity, flow-volume curves, closing volume, closing capacity, body plethysmograph functional residual volume and lung resistance to all lung volumes, static and dynamic pressure-volume curves, maximum flow static recoil curves, iso-volume pressure-flow curves, ventilatory and arterial blood gas studies at rest and exercise, lung lavage for cellular function and morphology, secreted proteins of macrophages and other constituents of alveolar wash fluid, technetium vascular scans, ^{133}Xe ventilation scans and ^{85}Gallium-citrate scans. When indicated, lung biopsy is done either through fiberoptic bronchoscope or via open thoracotomy. Tissue is studied by light microscopy, electron microscopy, culture (routine, fungal, mycobacteria and virus), collagen

synthesis and degradation (see Project # Z01 HL 02401-04 PB), electron probe analysis for mineral content and tissue culture (see Project # Z01 HL 02403-03 PB). Peripheral blood lymphocytes are isolated with dextran or Ficoll-Hypaque methods and are tested in vitro for antigenicity to constituents of the alveolar interstitium. Assays used include: migration inhibition factor, lymphocyte mediated cytolysis and monocyte chemotactic factor. Selected patients are entered into a drug treatment protocol where all patients are treated with corticosteroids (standard therapy) and 50% of the patients are treated (in addition) with azathioprine in a double blind fashion.

Major Findings: Completed studies in patients with IPF have demonstrated:

(1) Obstruction to airflow in the bronchiole is a significant part of the functional abnormalities in this disorder.
(2) The maximum transpulmonary negative pressure necessary to expand the lungs correlates well with the degree of interstitial fibrosis.
(3) The rate of decline of the arterial oxygen tension with increasing levels of exercise correlates with the extent of decrease shown histologically.
(4) Immune abnormalities associated with the collagen-vascular disorders are not frequently found in IPF.
(5) Ventilatory and arterial blood gas parameters with exercise are probably the most sensitive monitors of the disease process.
(6) Patients with IPF have marked abnormalities in ventilation-perfusion mismatching.
(7) Although the histopathology suggests increased amounts of collagen in the parenchyma of these patients, there is no biochemical evidence that confirm this impression. In fact, the biochemical data demonstrates normal concentrations of collagen in the IPF parenchyma.
(8) The rates of collagen synthesis/cell in biopsy specimens from patients with IPF are similar to control patients.
(9) Broncho-alveolar lavage fluid from patients with IPF have (compared to normals): a greater percentage of polymorphonucelar leukocytes and eosinophils, elevated levels of IgG but similar levels of the C4 and C6 components of complement. In comparison, patients with chronic hypersensitivity pneumonitis (CHP) have mostly lymphocytes in their broncho-alveolar lavage fluid. The respiratory lymphocytes in CHP have a T-cell to B-cell ratio of 15 to 1 (compared to their own peripheral blood T/B ratio of 3) suggesting the lung can act as an independent immune organ in this disease.
(10) ^{67}Gallium-citrate is taken up in the lungs of patients with IPF (65-70%) compared to controls (<5%). The amount of ^{67}Ga uptake correlates with the broncho-alveolar lavage and the histopathologic findings.
(11) Peripheral blood lymphocytes from >95% of patients with IPF recognize collagen as antigenic (compared to 0% of normals). In response, these lymphocytes produce migration inhibition factor and will lyse collagen-coated sheep red blood cells. Using anti-human-T-cell antiserum, it has been possible to demonstrate that the T-lymphocyte

is necessary to mediate these phenomena.

Preliminary studies in patients with IPF suggest:

(1) Peripheral lymphocytes from these patients will lyse human lung fibroblasts grown in culture.
(2) Tissue typing studies suggest that most patients with IPF have twice normal incidence of HLA-12, an antigen of the B histocompatibility locus.

Significance to Biomedical Research and Institute Program: The fibrotic lung disorders are almost uniformly fatal and affect a significant proportion of the population. Up to this time, there has been no information on pathogenesis and there is essentially no cure. By combining studies of patients with these disorders with our large basic research program concerning the control of connective tissue composition and accumulation in lung, we expect to make major inroads into understanding and eventually curing these disorders.

Proposed Course to Project: Studies as outlined will be continued. As methods are developed in the basic laboratory, they will be applied to study the biopsy specimens from human lung. Particularly important are the studies in lung explants and tissue culture where the manipulation and control of lung collagen synthesis and degradation can be studied with pharmacologic agents. Immunologic studies will continue to explore cell-mediated immune mechanisms in these disorders. As results with pharmacologic agents become promising, they will be studied in patients when applicable.

Publications:

Hance, A.J. and Crystal, R.G.: The connective tissue of lung. Amer. Rev. Resp. Dis. 112: 657-711, 1975

Fulmer, J.D., Roberts, W.C. and Crystal, R.G.: Diffuse fibrotic lung disease: a correlative study. Chest 69 (Suppl.): 263-265, 1976

Fulmer, J.D. and Crystal, R.G.: The Biochemical Basis of Pulmonary Function. In Crystal, R.G. (Ed.): The Biochemical Basis of Pulmonary Function. New York, Marcel Dekker, 1976, pp. 419-466

Crystal, R.G.: Biochemical Processes in the Normal Lung. In Bouhuys, A. (Ed.): Lung Cells in Disease. Amsterdam, North Holland-ASP Biological and Medical Press, 1976 (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02408-01 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Investigation of the Hereditary Disorders of Connective Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	J.A. McDonald	Research Associate	PB NHLI
OTHER:	J.D. Fulmer	Staff Investigator	PB NHLI
	N.A. Elson	Staff Investigator	PB NHLI
	R.S. Bienkowski	Staff Fellow	PB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.0	0.4

SUMMARY OF WORK (200 words or less - underline keywords)

The complexities of the anatomic arrangement of the lung together with the complexities of the biochemical composition of lung connective tissue, make it difficult to attempt to define how connective tissue controls lung structure and function. By studying the hereditary disorders of connective tissue, we are defining the biochemical defects at the molecular level and correlating these with the mechanical properties of lung defined by pulmonary function testing. Thus, by capitalizing on "experiments of nature" it is possible to make inferences of the biochemical-mechanical influences on lung structure and function.

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Project Description:

Objectives: The connective tissue of lung is complex, consisting of four genetically distinct types of collagen, elastic fibers (elastin together with microfibrils) and seven types of glycosaminoglycans with their associated proteins. This complexity together with the intricate anatomical arrangement of lung parenchyma makes the study of the influence of connective tissue on the control of lung structure and function extraordinarily difficult. The major objectives of this proposal are attempts to use "experiments of nature" involving connective tissue such that if we can understand the biochemical abnormalities of connective tissue in these hereditary disorders, we can then make inferences on the role each connective tissue component plays in the maintenance of lung structure and function. The lung is the only organ of the body where it is relatively easy using the sophisticated methodology of pulmonary function testing to make direct inferences on the role of connective tissue in controlling lung mechanical properties. Thus, the lung affords an opportunity for understanding the biochemical-mechanical correlates of connective tissue.

Specifically, the ongoing projects in this work are: (1) the development of tissue culture models for studying collagen synthesis in fibroblasts from patients with the hereditary disorders of connective tissue; (2) definition of the quality of collagen synthesized by fibroblasts in culture, e.g., relative hydroxylation of glycine residues, glycosylation of hydroxylysine, relative hydroxylation of proline residues, cross-links, types of collagen synthesized, and quantitation of collagen degradation; (3) to utilize methods to evaluate the types of glycosaminoglycans synthesized in culture as well as the rates of synthesis and degradation of these compounds; (4) to quantitate the rates of synthesis of elastin in these cultures and its associated cross-links; (5) to utilize these methods to specifically define the biochemical abnormalities in patients with disorders such as Marfan's Syndrome, Ehlers-Danlos Syndrome and Osteogenesis Imperfecta; and (6) to evaluate the same patients using sophisticated methods of pulmonary function including lung volumes, flow rates, body plethysmography, exercise studies with gas exchange parameters, closing volumes, flow-volume curves, maximum flow-static recoil curves, and ventilation-perfusion analysis with nuclear scanning methods.

This is a long term project but with the methodology available today, it should be possible to specifically define the role of each connective tissue component in lung and demonstrate how these components describe pulmonary abnormalities in these herediatry disorders.

Methods: Fibroblasts from patients with hereditary disorders of connective tissue and normal controls are grown from biopsies obtained by conventional methods. Cells are labeled with [^{14}C]lysine, [^{14}C]proline, [^{14}C]valine, [^{14}C]glucosamine or [^{35}S]sulfate in order to label collagen, elastin and/or glycosaminoglycans. The newly synthesized connective tissue components are then analyzed using previously described methods (see Project # Z01 HL 02401-04 PB, Project # Z01 HL 02403-03 PB and Project # Z01 HL 02406-03 PB).

In addition, carbohydrate moeities of connective tissue are labeled with [^{14}C]glucose to evaluate post-secretory removal of carbohydrates. Patients admitted to the clinical service of the Pulmonary Branch who have hereditary disorders of connective tissue are fully evaluated using methods described in Project # Z01 HL 02407-02 PB. The functional abnormalities derived from these studies are compared with similar studies from patients with other forms of lung disease and normals.

Major Findings: This work is just beginning and the past year has been spent primarily in developing methodologies to define the biochemical abnormalities of fibroblasts in patients with these disorders. The first question to be answered is whether fibroblasts derived from patients of different ages behave similarly in culture. Newly synthesized collagen of fibroblasts from seven normal controls ages 20 to 70 were virtually identical with respect to the amount of collagen synthesized, lysine hydroxylation and hydroxylysine glycosylation. The newly synthesized collagen in these cultures has more hydroxylysine than expected from extracted Types I and III collagen and the hydroxylysine in this newly synthesized collagen was glycosylated to a greater extent than extracted Types I and III collagen (see Project # Z01 HL 02403-03 PB and Project # Z01 HL 02406-03 PB). This finding suggests that: (1) other types of collagen (e.g., basement membrane collagen) are being synthesized by fibroblast cultures; (2) newly synthesized collagen differs from extracted collagen, with higher values of hydroxylation and glycosylation in the newly synthesized material which is then altered as collagen is laid down in the connective tissue matrix; or (3) fibroblasts do not reflect normal collagen synthesis. In order to answer these questions, rabbits are being labeled in vivo and their collagen is being analyzed to compare newly synthesized collagen in vivo with that synthesized by fibroblasts in culture. Basement membrane collagen from rabbits are being prepared for comparison with the material synthesized by fibroblasts in culture.

Preliminary studies with fibroblasts from one patient with Ehlers-Danlos Syndrome have demonstrated the relative rate of collagen synthesis compared to the synthesis of all other proteins in the culture is markedly high, even though there are normal quantities of lysine hydroxylation and hydroxylysine glycosylation. Preliminary analysis of skin fibroblasts from patients with scleroderma have thus far demonstrated no abnormalities in the parameters being evaluated.

Pulmonary function studies of these patients are ongoing, but as yet a pattern of disease groups has not been established.

Significance to Biomedical Research and Institute Program: The approaches being used will eventually define the molecular defects in the hereditary disorders of connective tissue. By understanding these defects in the same patients in which abnormalities in mechanical properties of lung function are defined will enable us to make significant inferences on the biochemical-mechanical effects of the complex connective tissue in lung.

Hopefully definition of these molecular defects will lead to improved diagnostic methods for genetic counseling, prognosis and eventually to rational therapy of these disorders.

Proposed Course to Project: As methods are being developed, we are seeking patients referrals to provide clinical material so that biochemical and functional studies may be done in the same patients. The basic questions raised by the differences seen between newly synthesized collagen in vitro and collagen laid down in vivo are being approached as outlined. Methods are being developed to evaluate not only collagen synthesis and degradation in the cultures but elastin and glycosaminoglycan synthesis and degradation as well. These in vitro tissue culture models are being studied to continue to define the biochemical abnormalities underlying various hereditary disorders of connective tissue and to learn to modify the types and amounts of connective tissue synthesized and degraded by these cells in culture. Definition of the normal processes of connective tissue synthesis and the effect of genetic abnormalities of it, should ultimately lead to the diagnostic and therapeutic approaches of these disorders. The understanding resulting from these studies should also be applicable to evaluating, defining and treating disordered connective tissue metabolism in diseases such as pulmonary fibrosis and emphysema.

Publications:

Hance, A.J. and Crystal, R.G.: The connective tissue of lung. Amer. Rev. Resp. Dis. 112: 657-711, 1975

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02409-01 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Degradation of Collagen in Lung

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.G. Crystal	Chief, Pulmonary Branch	PB NHLI
	R.S. Bienkowski	Staff Fellow	PB NHLI
	A.L. Horwitz	Research Associate	PB NHLI
	J.A. Kelman	Clinical Associate	PB NHLI
OTHER:	J.A. McDonald	Research Associate	PB NHLI
	K.H. Bradley	Chemist	PB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	2.7	1.6

SUMMARY OF WORK (200 words or less - underline keywords)

The adult lung continually synthesizes collagen but the total amount of collagen present remains constant. Thus, mechanisms must be present for the continued breakdown of collagen to match this rate of synthesis. Two mechanisms of lung collagen degradation are being evaluated: (1) extracellular degradation via collagenase secreted by alveolar macrophages, monocytes, polymorphonuclear leukocytes and fibroblasts; and (2) intracellular degradation by lung explants and fibroblasts. Both mechanisms appear opperant in lung and they may be critical control points in modulating the presence of lung collagen in normals, pulmonary fibrosis and emphysema.

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PHS-6040
(12-75)

Project Description:

Ojectives: The adult lung continually synthesizes collagen at a rate which represents 2 to 4% of the total proteins being synthesized at one time. However, the total amount of collagen in the adult lung remains constant. Therefore, there must exist mechanisms by which collagen is degraded. The objective of this project is to define, quantitate and learn to modify the mechanisms by which collagen is degraded in the lung.

Collagen is an unusual protein in that when it is in its native triple helical state there are only three proteases known which will degrade it. One is bacterial collagenase which is obviously not operant in the normal lung. The second is an acid cathepsin which is a lysozomal enzyme (described as yet only in liver) which acts at acid pH. The third is via animal collagenase, a neutral protease which acts in the extracellular milieu to degrade triple helical native collagen. It has a pH optimum in the range of 7 to 8, and it cleaves collagen at one site 75% of the distance from the N-terminus of the triple helical molecule. Once this cleavage has been made, the inherent instability of the collagen molecule is such that the triple helical structure cannot be maintained at body temperature, it denatures and then other proteases active in the extracellular milieu can degrade the remainder of the molecule.

Although previously uninvestigated as related to collagen, it is also possible that there are intracellular mechanisms of collagen degradation in the same fashion as intracellular mechanisms for degradation of intracellular enzymes. This is an attractive hypothesis since it might result in a mechanism by which the cell can control the destruction of "defective" collagen prior to its secretion.

The objectives of this project are to define the collagenases present in lung and to determine if intracellular mechanisms of collagen degradation are operant in lung cells.

Methods: Two model systems are being utilized to achieve these goals. In the first, explants of lung tissue (animal or human) are incubated in vitro with short pulses of [^{14}C] or [^{3}H]proline or lysine for up to 20 minutes. The tissue is then washed extensively with large amounts of unlabeled proline or lysine and the incubation is continued. Since hydroxyproline and hydroxylysine are formed after the protein is synthesized and since the collagen molecule is 140,000-150,000 molecular weight when it is first synthesized, then the finding of dialyzable labeled hydroxyproline or hydroxylysine is evidence of destruction of collagen. To determine whether this destruction is intracellular or extracellular, exogenous labeled collagen or serum can be added to the system. In addition, the kinetics of this degradation and the effect of agents such as colchicine can be determined.

In a variation of the same system, fibroblasts or other lung cells in culture can be evaluated in the same fashion. This method has advantages in that the kinetics of pulse-chase labeling are much faster and therefore, the system is cleaner. In addition, cells can be isolated and the presence of intracellular labeled hydroxyproline or lysine can be directly determined.

In the second system, the production of collagenase by lung explant, cultured lung cells, or alveolar macrophages obtained directly from the lung may be determined. In addition, collagenase production by purified blood elements such as monocytes and polymorphonuclear leukocytes can be evaluated. In these methods these cells are placed in culture and the media is collected at daily intervals. The collagenase activity is assayed using [^{14}C] collagen fibrils, [^{3}H]tropocollagen synthesized by lung cells in culture, extracted native collagen of different types, or segment long spacing aggregates using electron microscopic methods. These techniques can define lysis of the native tiple helical collagen and specifically define the anatomic site of cleavage in the collagen molecule.

Major Findings: Studies with the explant system have shown that lung explants in culture will produce a collagenase which will lyse extracellular native triple helical collagen. In addition, new mechanisms of collagen degradation, probably intracellular degradation, have been defined in explants from lung as well as from cultured lung fibroblasts. It appears that 25 to 30% of the collagen synthesized by lung explants is degraded intracellularly prior to its secretion from the cell. This phenomena is not affected by serum and is not due to proteases in the explant media secondary to artifacts of the model system. Preliminary studies with colchicine have shown that there may be an increase in intracellular degradation in the presence of colchicine. Since this drug is known to inhibit the secretion of collagen, it is possible that holding up of the secretion of the collagen molecule may allow these intracellular mechanisms to further degrade it. Studies with fibroblast cultures have demonstrated that they also rapidly degrade approximately 25% of the collagen synthesized and that these cultures can be shown to have labeled hydroxyproline within the cell suggesting again that intracellular degradation is taking place.

Studies with the collagenase assays have demonstrated that the pulmonary alveolar macrophage, human lung fibroblast, human blood monocyte, and human polymorphonuclear leukocyte all secrete a collagenase into the media when they are in culture. In the alveolar macrophage, this mechanism has been shown to be partially specific as related to the activation of collagenase. For example, in rabbits treated with BCG, their alveolar macrophages secrete more collagenase in culture than do alveolar macrophages from the normal rabbit. When the BCG animal macrophages are exposed to mycobacterium in vitro, they will secrete even more collagenase than alveolar macrophages from a normal animal when put under the same conditions. Most interestingly, it appears that the collagenase secreted by the alveolar macrophage is in a latent form that can be activated by a neutral protease

(molecular weight 13,000) also secreted by the same cell. Thus mechanisms have been described where collagen may be degraded either intracellularly or extracellularly and control mechanisms by which these processes are regulated are being evaluated.

Significance to Biomedical Research and Institute Program: The control of the connective tissue of lung is fundamental to an understanding of the structure and function of this organ. In fibrotic disease and in emphysema there are apparent abnormalities of connective tissue. In fibrotic disease, the excess of collagen (at least at a local level) may be modulated by the use of mechanisms which will break down this scar tissue. In the emphysematous disorders, the destruction of connective tissue may be modulated by proteases such as collagenase present in lung. An investigation of these processes and an understanding of how they may be controlled is vital to the understanding and eventual therapy of a major source of lung disease.

Proposed Course to Project: We are continuing to investigate the mechanisms of intracellular degradation as this may afford a critical control step which may be modulated in lung disease. It is possible that pharmacological agents, particularly those that inhibit the secretion of collagen, may be useful in terms of controlling intracellular collagen degradation. We are continuing to evaluate the cells of the lung that make collagenase and the mechanisms by which collagenase is secreted, activated and/or inhibited. These methods will be evaluated in animal models, tissue culture and alveolar wash material from human lungs of patients who are normal as well as those with significant pulmonary disease.

Publications:

Hance, A.J. and Crystal, R.G.: The connective tissue of lung. Amer. Rev. Resp. Dis. 112: 657-711, 1975

Horwitz, A.L. and Crystal, R.G.: Collagenase from rabbit pulmonary alveolar macrophages. Biochem. Biophys. Res. Comm. 69: 296-303, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02501-03 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Synthesis, Uptake and Storage of Biogenic Amines in Rat Lung

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. E. Shaff	Chemist	PB NHLI
OTHER:	M. A. Beaven	Pharmacologist	PB NHLI
	Z. Horakova	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.1	0.7

SUMMARY OF WORK (200 words or less - underline keywords)

Studies with rat lung slices indicated that serotonin, but not histamine, is taken up by a mechanism with the characteristics of active transport. The uptake is inhibited by metabolic poisons such as N-ethylmaleimide, iodoacetate, by low temperature (0°C) and by compounds known to block transport of serotonin into neurones and platelets as, for example, desmethylimipramine and the Lilly compound 110140. The uptake is saturable ($K_m \sim 5 \times 10^{-7}$), rapid ($t_{1/2} \sim 5$ min) and relatively specific. Tyramine and, to a lesser extent, tryptamine compete for the uptake, but N-acetylserotonin, 5,6-dihydroxytryptamine, norepinephrine and histamine do not. The labeled serotonin is uniformly distributed into endogenous stores; reserpine was found to release endogenous and labeled serotonin from the slice in equal proportions, i.e. the specific activity of residual serotonin in the slice was unchanged. Since destruction of serotonergic and adrenergic neurones by 6-hydroxydopamine and 5,6-dihydroxytryptamine did not affect uptake, the stores were non-neuronal. As indicated by histological fluorescence studies, serotonin is present in mast cells located exclusively around small blood vessels.

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Project Description:

Introduction. Our previous studies in rat lung (see Project Report Z01 HL 02501-02 PB) have shown that the level of biogenic amines are low (< 0.1 µg/g) at birth but increase with age. The increase in histamine levels continues throughout life and is enhanced by infection. The serotonin levels increase sharply around the 3rd-4th week of life to 4-6 µg/g and then stay constant for the remainder of the animal's life. Although it is thought that in rodents both serotonin and histamine are stored in mast cells and that both amines serve the same function (as mediators of inflammation, etc.), our studies have shown that in lung the two amines have quite different characteristics. In young rats, serotonin appears to have a rapid turnover. Histamine is synthesized very slowly. Serotonin but not histamine is depleted by reserpine. Compound 48/80 partially depletes lung of histamine but not of serotonin. Lung serotonin levels actually increase after treatment with compound 48/80, due to uptake of serotonin released into the circulation from peripheral organs. These findings led us to the conclusion that histamine and serotonin were stored in different cells, and histological studies indicated that serotonin was stored exclusively in mast cells located in the intima of small blood vessels. The location of histamine was undetermined, but studies by other workers indicate that mast cells are also located along the bronchi and pleural membranes of the lung.

In this year's study, the mechanism of uptake and storage of serotonin in lung has been studied with lung slices. The uptake mechanism shows similar characteristics to that for serotonin in neurones.

Methods Employed. Rat lung slices (∿ 100 mg) were prepared with a Stadie-Riggs microtome and incubated in 2 ml Tyrode solution at 37° in flat-bottomed flasks. A monoamine oxidase inhibitor (Pargyline) was included in the mixture to prevent metabolic destruction of the serotonin. Drugs and labeled histamine or serotonin were added to the medium and aliquots of the medium removed at appropriate intervals for radioassay of the amines and their metabolites as described in previous reports. Release of labeled amines was studied by preincubating the slice with labeled amine for 30 min. The slice was briefly washed in Tyrode Ringer and transferred to fresh medium.

Results. Studies with lung slices *in vitro*. Tritiated serotonin is rapidly taken up into lung slices by saturable process (K_m ∿ 5 x 10^{-7} M) that is temperature dependent and which is inhibited by metabolic inhibitors such as N-ethylmaleimide, iodoacetate and cyanide. The uptake was blocked by desmethylimipramine and Lilly No. 110140, which are known to block uptake of serotonin into serotonergic neurones and blood platelets. Serotonin uptake was observed in lung slices of both adult and young (10-day-old) rats, although in the latter the levels of endogenous serotonin were low (< 0.1 µg/g). This and our earlier results suggested that the transport mechanism for serotonin is present at an early stage of life but that the mechanism for storage of serotonin is acquired only in later life (∿ 3-6 weeks of age). The uptake of serotonin was rapid. By 15 min a concentration gradient of 1:4:5 (medium to slice ratio) was obtained and by 30 and 60 min the ratio

was 1:8 and > 1:20 respectively. The uptake was evident in animals pretreated with 6-hydroxydopamine and 5,6-dihydroxydopamine (75 mg/kg i.p.), agents which are known to destroy adrenergic and serotonergic neurones.

Other characteristics of the serotonin uptake system were as follows. The uptake was inhibited competitively by tyramine ($K_i \sim 10^{-4}$ M) and to a lesser extent tryptamine ($K_i \sim 3 \times 10^{-4}$ M). N-Acetylserotonin, 5,6-dihydroxytryptamine, 6-hydroxydopamine did not influence the uptake in concentration up to 2.5×10^{-3} M.

When lung slices were incubated with the ^{3}H-serotonin and then transferred to fresh medium, the serotonin level declined in the tissue exponentially. Analysis of the kinetics of these reactions indicated that the $t_{1/2}$ for the uptake of serotonin was ∿ 5 min and for the release about 80 min.

An important experiment was the demonstration that reserpine released endogenous and labeled serotonin from lung slice in equal properties, i.e. specific activity of residual serotonin in lung was unchanged. This experiment indicated that the labeled serotonin taken up by the slice was uniformly incorporated into endogenous stores of the amine.

Chromatographic studies revealed that almost all (> 90%) of the labeled material in the lung was unchanged serotonin, whereas N-acetylserotonin appeared in increasing amounts in the medium as the levels of labeled serotonin declined. By 3 hr the label remaining in the medium was largely N-acetylserotonin. When the slices were transferred to fresh medium or when serotonin was released by reserpine, the label appearing in the medium was exclusively N-acetylserotonin or, when pargyline was omitted from the incubation medium, as 5-hydroxyindole acetic acid and N-acetylserotonin.

In contrast to serotonin, ^{3}H-histamine was not taken up by rat lung slices. Passage of histamine into the slice was slow (the ratio of histamine in medium to slice did not exceed 1) and was unaltered by incubation of the slice at 0°. Addition of serotonin to the medium did not affect the passage of histamine into the tissue nor did histamine alter the uptake of serotonin.

Pharmacological studies. Additional studies with L-histidine loading (500 mg/kg i.p.) failed to show any increase in histamine levels in lung in young and old rats upon loading (see last year's report). In contrast to serotonin, where administration of tryptophane and 5-hydroxytryptophane increase pulmonary levels of serotonin, histamine appears to be formed at a finite and very slow rate in lung. These and previous results suggest that lung histamine does not turn over but gradually accumulates with age. This finding is consistent with reports of Riley, West and others that mast cells slowly increase in number during development from infancy to the adult.

General conclusions and proposed course of work. Although it is generally assumed that serotonin and histamine coexist in mast cells of rodents, cattle and certain other species, the two amines appear to have quite different storage and release characteristics in rat lung. It would appear that

histamine is preformed, stored and is capable of rapid release in response to antigens to which the individual is sensitive or to nonspecific stimuli such as inflammatory reactions. Serotonin, on the other hand, is stored exclusively in mast cells around small blood vessels, it has a rapid turnover and is in equilibrium with serotonin in the circulation. Serotonin may have a continuing rather than intermittent role in the lung, possibly in the vasculature system. The possibility that the serotonin stores in lung can be uniformly labeled with tracer amounts of ^{3}H-serotonin will be explored in studies *in vivo*.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02502-03 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Inhibition of Tumor Growth in Mouse by Anti-inflammatory Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. A. Beaven	Pharmacologist	PB NHLI
	V. Hial	Visiting Fellow	HE NHLI
OTHER:	Z. Horakova	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)
Dr. V. Hial, Visiting Fellow, Hypertension-Endocrine Branch, National Heart and Lung Institute, Bethesda, Maryland 20014

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

SUMMARY OF WORK (200 words or less - underline keywords)

Oral daily administration of aspirin or indomethacin retarded growth of experimental tumors in mouse. Aspirin treatment, 150 mg/kg twice daily, inhibited growth of a transplantable mast-cell ascites tumor (P815) by 39-43% ($p < 0.001$) and of a subcutaneously transplanted Lewis lung carcinoma by 52% ($p < 0.025$) without adversely affecting body growth. The total serotonin, histamine and histidine decarboxylase content of the ascites tumor was also reduced as was the urinary excretion of the amines. Treatment with 3 and 5 mg/kg indomethacin resulted in 40 ($p < 0.01$) and 80 ($p < 0.001$) % reduction, respectively, in ascites tumor growth. With the higher dose of indomethacin, no tumor was observed in half of the animals inoculated with tumor, although signs of indomethacin toxicity (reduced body growth, gastric lesion) was evident in the animals.

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Introduction. The present work arose because of the findings of others that salicylates inhibit nucleotide synthesis and growth of bacteria and the mammalian fetus (Schwartz and Mandel, Biochem.Pharmacol. 31: 771, 1972; Janakidevi and Smith, J.Pharm.Pharmacol. 22: 51, 58 and 249, 1970) and that aspirin and indomethacin reduce the size and incidence of metastases of two experimental bone resorbing tumors in animals (Tashjian et al., Prostaglandins 3: 515, 1973; Powles et al., Brit.J.Cancer 28: 316, 1973). These findings led us to the question of whether salicylates and similar anti-inflammatory drugs inhibit metabolic activity and growth of rapidly growing tissues such as tumors. It is hoped ultimately to extend these studies to tissues with rapid cell turnover, such as stomach, and tissues undergoing pathological changes. Our preliminary studies showed that aspirin in pharmacological doses inhibits growth of two transplantable tumors in mouse, the mast-cell ascites tumor of Dunn and Potter and solid Lewis lung carcinoma (see last year's report Z01 HL 02502-02 PB). The present work extends these findings and shows that indomethacin as well as aspirin inhibit tumor growth *in vivo* and that all the salicylate-like anti-inflammatory drugs inhibit metabolic activity and growth of tumor cells in culture. This report describes the studies *in vivo*. The *in vitro* studies are described separately in the next report (Project No. Z01 HL 02520-01 PB).

Methods. Six-week-old CD_{f1} mice were obtained from the Division of Research Resources, N.I.H., and were housed in isolators under controlled conditions of lighting and temperature. The ascites tumor was transplanted intraperitoneally weekly. The Lewis lung tumor was transplanted by a no. 13 trocha needle subcutaneously into the flank of the animal. Tumors were removed and weighed after 7 days in the case of the ascites tumor and 14 days for the lung tumor. Drugs were given twice daily as a suspension by mouth. Histamine and serotonin were assayed by the enzymatic radioassays described in previous reports. Details of these procedures were given in last year's report (Z01 HL 02502-02 PB).

Findings. Treatment with aspirin, 150 mg/kg twice daily, reduced growth of the ascites tumor by 40% ($p < 0.007$) and that of the Lewis lung carcinoma by 52% ($p < 0.025$) (Table). This dose of aspirin did not impair growth or produce gastric lesions. Indomethacin was more effective than aspirin in inhibiting tumor growth. In doses of 1 mg/kg twice daily, growth of the ascites tumor was inhibited by 40% ($p < 0.01$) and after 5 mg/kg twice daily by 77% ($p < 0.001$) (Table). The higher dose of indomethacin impaired body growth (Table) and produced gastric lesions, however.

The reduction in ascites tumor growth was evident both from a diminution in volume and cell numbers but also from a 36-55% reduction in urinary excretion of histamine and serotonin. These amines are produced in large amounts by this tumor. The level of amines in tumor was unchanged and the reduced excretion of amines appared to reflect the decreased growth of the tumor.

Despite the reduction in ascites tumor volume, neither aspirin nor indomethacin treatment prolonged the life of the inoculated mice. In one study with aspirin in which mice ($n = 30$) were inoculated with 1.8×10^6 cells, all

mice, aspirin- and vehicle-treated, died within 48 hr (day 9 and 10). In another study in which mice (n = 20) received 100-fold fewer cells (2×10^4), all animals died on day 11 and 12. With indomethacin treatment, 3 and 5 mg/kg, the mice died on day 8, 9 and 10 as did the control mice. Examination of the abdominal contents revealed widespread bleeding into the abdominal cavity. Irrespective of tumor size, the animal showed similar symptoms at death. Further investigation of the effect of the drugs on survival and the mechanism of death has not been undertaken.

Significance and proposed course of project. The studies indicate that anti-inflammatory drugs possess anti-tumor activity. This possibility will be explored with individuals in the National Cancer Institute. See also comments in the following report (Project No. Z01 HL 02520-01 PB).

Publications:

Hial, V., Horakova, Z., Shaff, R.E. and Beaven, M.A.: Alteration of tumor growth by aspirin and indomethacin: Studies with transplantable ascites tumor and Lewis lung carcinoma in mouse. Eur.J.Pharmacol., in press.

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Table

Comparison of the effect of aspirin and indomethacin treatment on growth of ascites tumor and Lewis lung carcinoma

Values are mean ± SEM. Drugs were given twice daily. Treatment was begun 24 hr before inoculation with tumor.

Treatment	*(n)*	Final body weight		Tumor volume		% Reduction	No. of mice without tumor
		Control	Drug-treated	Control	Drug-treated		
		(g)		*(ml)*			
*Ascites tumor**							
Aspirin, 150 mg/kg	(44)	23.3 ± 0.3	21.6 ± 0.3 *p<0.001*	1.8 ± 0.1	1.1 ± 0.08	40 *p<0.001*	0/44
Indomethacin, 3 mg/kg	(19)	26.1 ± 0.6	25.8 ± 0.7 *NS*	1.3 ± 0.1	0.8 ± 0.13	40 *p<0.01*	2/19
" 5 mg/kg	(11)	26.9 ± 0.5	22.2 ± 0.8 *p<0.001*	1.5 ± 0.1	0.35 ± 0.1	77 *p<0.001*	5/11
*Lewis lung carcinoma***							
Aspirin, 150 mg/kg	(10)	22.3 ± 0.5	24.0 ± 0.4 *NS*	0.27 ± 0.06	0.13 ± 0.03	52 *p<0.025*	0/10

*Tumor was removed 7 days after inoculation.

**Tumor was removed 15 days after implantation.

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PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Disorders of Amine Metabolism in Human Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. A. Beaven	Pharmacologist	PB NHLI
	Z. Horakova	Pharmacologist	PB NHLI
OTHER:	G. Wilcox	Clin. Associate	HE NHLI

COOPERATING UNITS (if any) Dr. Gilbert Wilcox, Hypertension-Endocrine Br., NHLI; Dr. Allen Kaplan, Lab. Clin. Invest., NIAID: Dr. Stephen Katz, Dermatology Br., NCI; Dr. Morton Grossman, VA Hosp., Center of Digestive Diseases, Los Angeles, Calif.; Dr. Stephen Baylin, Lab. Clin. Metab., Johns Hopkins, Baltimore, Md., is a recipient of a special USPHS career development award; Dr. Marvin C. Gershengorn,

LAB/BRANCH Clin. Branch, NIAMDD.
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.6	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

Measurement of histaminase activity in fine needle biopsy aspirates of thyroid indicated that such a measurement provided a reliable and simple test for medullary carcinoma of the thyroid. The enzyme was present in high levels in aspirates drawn from this tumor but not in those drawn from other thyroid tumors or normal thyroid tissue. Studies in 17 patients with mastocytosis, basophilic leukemia, gastric carcinoma and cold-induced urticaria showed a general correlation in plasma histamine levels, urine histamine excretion and symptoms. A urine excretion of > 300 µg/24 hr (normal 17 µg) was accompanied by detectable levels of histamine in plasma and a high basal gastric secretion. Patients with values < 300 µg/24 hr had no detectable histamine in plasma, normal gastric secretion but had other symptoms associated with histamine release, such as pruritus, flushing, etc. Although patients with mastocytosis had elevated urine histamine, the values were lower than those with basophilic leukemia. This was attributed to 1) more immediate access of histamine in the basophil to the blood stream and 2) more rapid turnover of histamine in basophils than in the tissue mast cell.

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Introduction. An important objective of this section is the development of new assays which facilitate the study of disorders associated with the storage and metabolism of biogenic amines. The principal new procedures are listed in Table 1. These procedures permit direct measurement of the amines and enzymes in body fluids and biopsy samples at levels that could not be detected by the older fluorometric procedures. The major clinical findings obtained with these assays are also given in Table 1.

This year's main activity has been the measurement of histaminase (diamine oxidase) activity in thyroid biopsy specimens for the diagnosis of medullary thyroid carcinoma and further studies of histamine release in patients with cold- and cholinergic-induced urticarias. New procedures were also developed for the assay of dopamine-β-hydroxylase (DβH) and the amino-acid decarboxylases as described below.

Procedures. The principles of the procedures are outlined in Table 1 and have been described in detail in the annual reports for 1971 through 1975. The two procedures introduced this year, the tritium release assay for DβH and the micro-procedure for the measurement of $^{14}CO_2$ release from carboxyl labeled amino acids, are described below.

The DβH assay is based upon the measurement of tritiated water released from dopamine-β-β-^{3}H during hydroxylation by DβH. Chromatographic studies of the stoichiometry of this reaction indicated that hydroxylation resulted in the release of one tritium atom for each molecule of norepinephrine formed. Studies with specific enzyme inhibitors also showed that DβH was the only enzyme in rat and human serum capable of inducing tritium release. The optimum conditions for the assay, the requirements for inactivators of serum DβH inhibitors, the effect of drugs and inhibitors are described in detail in reference 6, Table 1. The procedure is similar to that for the histaminase assay--tritiated water is sublimed directly from the incubation mixture in Thunberg tubes. The assay is simple (other procedures require a series of enzymes and chemical reactions before isolation of the product), reproducible and sufficiently sensitive to measure the low activity in rat serum.

The micro-modification for the measurement of $^{14}CO_2$ release. Homogenates (10 or 25 µl) of tissue or biopsy specimens are incubated with an equal volume of reagent (50 nCi [2.5×10^{-4} M] of the carboxyl labeled L-amino acid, appropriately diluted with unlabeled amino acid; pyridoxal phosphate 10^{-5} M; and phosphate buffer, 0.1 M, pH 6.8) in an Eppendorf vial enclosed within a 20 ml screw-cap counting vial. Twenty microliters of Hyamine hydroxide solution is placed in the bottom of the counting vial to trap $^{14}CO_2$. The vials are incubated in the usual manner and when the reaction is complete, the Eppendorf vial is removed and discarded. Scintillation fluid is added directly into the counting vial. Studies with $NaH^{14}CO_3$ indicated that the loss of $^{14}CO_2$ by use of the screw caps was negligible (< 2%). This procedure avoids 1) the need to transfer Hyamine for radioassay, 2) the need for large amounts of Hyamine which tend to make the incubation mixture alkaline, 3) the use of rubber septums and cups and 4) allows measurements on a micro scale (this was not possible with the previously described procedures).

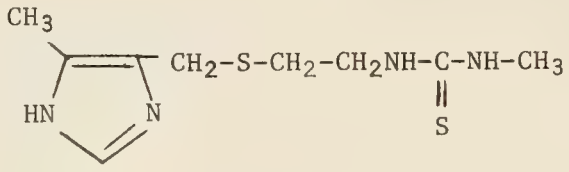
Clinical studies. Medullary carcinoma of the thyroid (with Dr. Marvin Gershengorn). Fine needle aspiration biopsy has been introduced recently for the diagnosis of a variety of thyroid disorders but has not been applied in the diagnosis of medullary thyroid carcinoma. Our previous studies have shown (Amer.J.Med. 53: 723, 1973; Ann.Int.Med. 78: 561, 1973) that this tumor consistently contains high levels of histaminase. Since the tritium release assay of histaminase is simpler to perform than the radioimmunoassay of calcitonin (another biochemical marker for this tumor), the usefulness of the release assay in detecting tumor in thyroid biopsy specimens was assessed.

Histaminase activity in two biopsy specimens of medullary carcinoma (550-660 units/g) was 12-300 times higher than that in thyroid biopsy specimens from patients with benign and malignant follicular adenomas (2-44 units/g, n = 17), metastatic melanoma (to thyroid) (9 units/g, n = 1) and goiter (12 units/g, n = 1) and 30 times higher than that in normal thyroid tissue (10-60, mean 17, units/g, n = 9). The presence of medullary carcinoma was confirmed by cytological examination of the biopsy and later of the surgical specimens. In most cases, the assay was performed on mg samples and was completed within 45 min. Currently the calcitonin assay requires up to one week for completion. A wide variety of tumors (n = 60) have been screened for histaminase activity by the tritium release assay and to date in only two, medullary thyroid carcinoma and the small-cell carcinoma of the lung (Oates cell)(Baylin et al., N.Engl.J.Med. 293: 1286, 1975), has this enzyme been found in high levels.

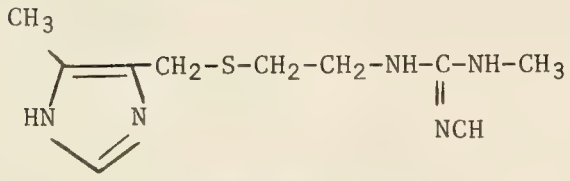
Patients with histamine producing tumors. From the patients studied at the NIH as well as those referred to us from outside clinics, the following patterns have emerged. The urinary excretion of histamine was consistently high in patients with *acute basophilic leukemia* and in one patient as high as 1400 µg/24 hr (versus 17 µg/24 hr for normal subjects (Table 2). Blood histamine levels in these patients ranged as high as 30 µg/ml (versus 0.1 µg/ml for normal subjects). Two patients with symptoms of hyperhistaminemia (peptic ulcers, wheezing and urticaria) also had significant quantities of histamine in plasma (up to 47 ng/ml). Treatment with the H_2 inhibitor, metiamide, alleviated the symptoms in both patients but did not alter the course of the disease. As noted by Code and other workers, there was not always a precise correlation between basophilic count and histamine level, although both were markedly elevated. The histamine content per basophil was low compared to that in normal subjects as was the number of granules per basophil.

Of the 10 patients studied with *mastocytosis*, the urinary histamine levels were lower and ranged from high normal levels (30 µg/24 hr) to 230 µg/24 hr. The three patients with values over 200 µg/24 hr had the systemic variety of the disorder. The other patients either had cutaneous lesions or the extent of involvement of other organs was unknown. In all patients blood histamine levels were normal. With the help of Dr. Katz, patients with classical macular urticaria pigmentosa without systemic involvement are now being located to determine whether measurement of urinary histamine levels can be used to distinguish patients with the systemic form of the disease from those whose disease is still confined to skin.

In addition to the two patients with basophilic leukemia, a high plasma histamine level has been observed in a patient with *gastric carcinoma* (a patient of Dr. Grossman) who was first studied last year. This patient continued to have detectable histamine levels in plasma, a markedly elevated urinary histamine excretion (453 µg/24 hr compared to 370 µg/24 hr last year) and an uncomfortably high basal gastric secretion (over 1 L/hr). Treatment with metiamide (I) has abolished the gastric secretion without altering the plasma histamine levels. He recently developed agranulocytosis, and the drug therapy was discontinued. Similar complications with this drug have been observed in clinical trials in England. As a result of these reports, S.K.F. Ltd. has introduced a third H_2 inhibitor, cimetidine (II), which is devoid of the thio-urea moiety, the group thought to be responsible for the agranulocytosis. Hematopoietic toxicity has not been detected in dogs and thus far in man with the new drug.



Metiamide [I]



Cimetidine [II]

A summary of all histamine measurements made to date is given in Table 2. From these data, several conclusions may be drawn. All 3 patients with a urinary excretion above 300 µg/24 hr (the two patients with basophilic leukemia and the one with gastric carcinoma) had detectable levels of histamine in plasma and unusually high gastric acid secretion. Patients with urine histamine excretion below 300 µg/24 hr did not have detectable (< 1 ng/ml) histamine in plasma nor a high gastric secretion. The patients with elevated urinary histamine excretion did have other symptoms associated with histamine release, such as periodic flushing, hypotension, pruritus, urticaria and headaches. Another observation is that patients with mastocytosis, even those with widespread involvement, did not excrete as much histamine as patients with basophilic leukemia. This may be due to different exposure of newly released histamine to degradative enzymes or to differences in the turnover

and storage of histamine in the mast cell and basophil. In granulocytic leukemia, the basophils are large, diffusely granulated, and have a relatively low histamine content (compared to normal basophils). In mastocytosis, the mast cells, although increased in number, are normal looking and have a normal histamine content.

Studies in patients with cold- and exercise-induced urticaria. Additional patients have been located and studied this year. Exposure of one arm to ice-cold water or vibration resulted in the appearance of histamine in plasma in the brachial vein (an increase from < 1 to 8-40 ng/ml) and in a few patients in plasma of the systemic circulation. The latter patients experienced marked hypotension and faintness. All patients showed the familiar itching, redness and edema of the exposed arm. There was a close correlation between the development and subsidence of symptoms with the rise and fall in plasma histamine levels. In two patients the onset of symptoms was delayed 15-20 min as was the appearance of histamine in plasma. Such release is not observed in control patients. The disorders appear to be mediated by a circulating antibody. Isolation of the IgE fraction from the affected patients and transfer to a normal subject resulted in histamine release and the appearance of symptoms in the normal subject upon exposure to cold. These studies are the first to show precise correlation between histamine and development of symptoms in human subjects and illustrate the utility of the enzymatic isotope assay for histamine.

In further work, a simple blister test has been devised to identify patients with the cold-induced urticaria. In this test an ice cube is placed upon a mechanically induced blister and the histamine content of the blister fluid measured. Histamine levels in blister fluid of nonaffected individuals ranged from 2.0 to 6.1 ng/ml (n = 4) and in affected individuals from 41 to 128 ng/ml (n = 8).

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Table 1

Radioassay of Biogenic Amines and Enzymes and their Clinical Applications

Assay	Type of Assay	Reference	Principal Application
Amines			
Histamine	Enzymatic formation of ^{14}C-methyl-metabolites using S-adenosylmethionine-^{14}C methyl as methyl donor	1,2,3	Release of histamine and serotonin cold- and exercise-induced urticarias (7) and certain tumors (see this report)
Serotonin		2	
Catecholamines		Work in progress	
Amino-acid decarboxylases			
Histidine-	Release of $^{14}CO_3$ from carboxyl labeled amino acid	See this report	Discovery of high DOPA decarboxylase levels in med. carcinoma of thyroid (8) and ornithine decarboxylase in thymus lymphocytes (9)
DOPA-			
Ornithine-			
Amine oxidases			
Monoamine oxidase	α^{14}C-Benzylamine to ^{14}C-benzaldehyde	4	Discovery of high histaminase activity in med. carcinoma of thyroid (10) and Oates cell carcinoma (see this report). Early detection of disorders of pregnancy (11)
Diamine oxidase (histaminase)	^{3}H-release from β-^{3}H-histamine	5	
Hydroxylating enzymes			
Dopamine-β-hydroxylase	^{3}H release from dopamine-β-^{3}H	6. See this report	Utility demonstrated in human serum (6)
Methylating enzymes			
Catechol-O-methyl-transferase	Formation of ^{14}C-methyl-metabolites with SaMe-^{14}C as methyl donor	2,3	---
Histamine-N-methyl-transferase			
Hydroxyindole-O-methyl-transferases			

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Table 2

Histamine Content of Body Fluids under Normal and Pathological Conditions

		Urine (µg/24 hr)			Whole Blood (µg/ml)		Plasma (µg/ml)	
		n	mean	range	mean	range	mean	range
Normal	male	19	16	< 5-51	0.079	0.03-0.158	< 0.001	---
	female	9	19	< 5-92				
	children (< 12 yr)	6	8	< 5-20				
Mastocytosis	systemic, proven	3	209	202-231	--		< 0.001	
	cutaneous and unknown involvement	7	68	30-116	--		< 0.001	
Basophilic leukemia		6	861	323-1400	7.5	0.6-30.1	0.049	
Gastric carcinoma		1	368,453		--		0.004,0.008	
Anaphylactic reaction (yellow jacket sting)		1	59 µg/12 hr		--		---	
Miscellaneous diseases (Migraine, Oates cell carcinoma, Dengue's Fever, Eosinophilia, etc.)		18	22	< 5-88	0.080	0.056-0.092	< 0.001	

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02507-03 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Spin Label Studies of Acetylcholinesterase and the Acetycholine Receptor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. Wee	Visiting Fellow	PB NHLI
OTHER:	C. F. Chignell	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)

Dr. P. W. Taylor, Department of Pharmacology, University of California, San Diego, La Jolla, California 92037

LAB/BRANCH

Pulmonary

SECTION

Molecular Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

The topography of the active site of acetylcholinesterase and the acetylcholine receptor from Torpedo californica has been examined with the aid of a mononitroxide analog (I) of decamethonium and symmetrical dinitroxide analogs (II,III) of decamethonium and hexamethonium. The I_{50} values for the inhibition of acetylcholinesterase by labels I-III were 0.25 μM, 0.18 μM and 3.3 μM respectively. Electron spin resonance (ESR) measurements indicated that the decamethonium dinitroxide label II bound to acetylcholinesterase in an extended conformation. The ESR spectrum also contained two highly immobilized components suggesting that each quaternary nitrogen experienced a different environment. These findings have provided direct proof for a two-point attachment of decamethonium analogs to acetylcholinesterase involving the active site and a peripheral anionic site.

Label I exhibited the highest affinity for the acetylcholine receptor from which it was quantitatively displaced by a stoichiometric amount of α-bungarotoxin. The affinity of I for the receptor increased threefold during a 30-min incubation. These findings suggest that the desensitization phenomenon observed for nicotinic agonists may result from a time-dependent increase in the affinity of these agents for the receptor.

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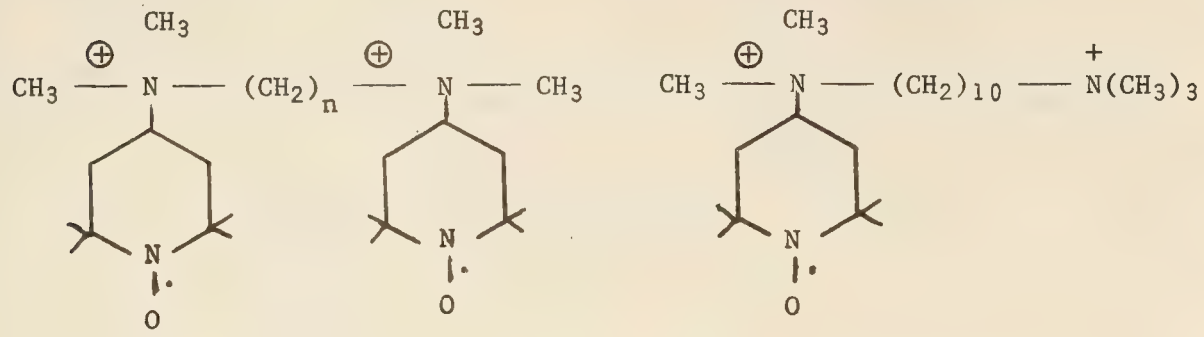
It is the aim of this study to examine the topography of acetylcholinesterase and the acetylcholine receptor from *Torpedo californica* with the aid of three spin-labeled bisquaternary ligands (I-III).

III n = 6
II n = 10



I

Acetylcholinesterase was isolated from the electroplax of *Torpedo californica* by previously published methods (Mol.Pharmacol. 10: 78, 1974). Membranes enriched in cholinergic receptor were obtained by the differential centrifugation, density gradient centrifugation and affinity partition procedure described by Flanagan and co-workers (J.Biol.Chem., 1976, in press). Electron spin resonance (ESR) measurements were made with a Varian E-4 spectrometer equipped with a quartz aqueous sample cell.

Acetylcholinesterase from *Torpedo californica* is a tetrameric enzyme with a molecular weight of 320,000. Each subunit contains a single active site which can hydrolyze acetylcholine. The spin-labeled bisquaternary ligands were inhibitors of acetylcholinesterase (Table 1). Replacement of a single quaternary methyl group in decamethonium by the nitroxide bearing piperidine ring to give spin label I resulted in a 16- to 20-fold increase in the affinity of the ligand for acetylcholinesterase. The substitution of a second quaternary methyl group in label I by a piperidine moiety resulted in only a 1.4- to 1.8-fold increase in affinity (Table 1). The I_{50} values for inhibition of enzymatic activity were close to the K_D values derived from direct measurement of binding by ESR (Table 1), suggesting that the spin labels did indeed bind to the active site of acetylcholinesterase.

In the presence of acetylcholinesterase, the ESR spectrum of label II became highly immobilized ($2T_{\prime\prime}$ = 70 gauss). The apparent signal intensity of enzyme bound II increased twofold. These findings indicate that label II binds to acetylcholinesterase in an extended conformation in which both ends of the molecule are firmly attached to the enzyme. It had been previously suggested that bisquaternary ammonium ligands in which the maximal distance between the two quaternary nitrogens was 14.5 A bound to acetylcholinesterase via a two-point attachment in which one of the cationic groups interacted with an anionic site at the active site while the other was associated with a peripheral anionic site (Belleau et al., Mol.Pharmacol. 6: 41, 1970; Roufogalis and Quist, ibid 8: 41, 1972). However, these conclusions were based on kinetic studies which provided only indirect evidence while our findings provide direct evidence for such a binding model. A close examination of the ESR spectrum of

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II bound to acetylcholinesterase revealed the presence of two bound species which may represent a different mode of interaction between the two quaternary nitrogens and the active and peripheral sites.

When label I bound to acetylcholinesterase, its ESR spectrum became broad and asymmetric with a maximal hyperfine splitting ($2T_{\prime\prime}$) of 70 gauss. In contrast to label II, the signal intensity of spin label I did not change when this inhibitor bound to acetylcholinesterase. Methanesulfonylation of the active site serine of acetylcholinesterase reduced the affinities of labels I and II by 6- and 16-fold, respectively (Table 2). These findings suggest that in the unmodified enzyme label I is preferentially oriented with the piperidine moiety at the active site.

The affinity of label III for acetylcholinesterase was 13-fold lower than that of label II (Table 1). Methanesulfonylation of acetylcholinesterase reduced the affinity for label III to the point where it became impossible to detect binding by ESR. The ESR spectrum of label III bound to acetylcholinesterase was also characteristic of a highly immobilized spin label ($2T_{\prime\prime}$ = 70 gauss). While there was an apparent 3-fold increase in the intensity of enzyme-bound III, there was no evidence for the presence of more than one bound species. This would suggest that label III binds to acetylcholinesterase in an extended conformation in which at least one of the quaternary nitrogens is located at the active site. Since both ends of label III are anchored firmly and yet the second quaternary nitrogen cannot reach the peripheral anionic site, it would appear that there may be accessory (hydrophobic?) sites between the active and peripheral sites.

In the presence of cholinergic receptor enriched membranes from *Torpedo californica*, the ESR spectrum of spin label I became broad and asymmetric with a maximal hyperfine splitting of 70 gauss. The wide separation between the outer hyperfine lines is indicative of a bound nitroxide that is highly immobilized on the receptor surface. Titration of the membranes with label I clearly showed that there was one spin label binding site for each cobra α-toxin binding site. The addition of a stoichiometric amount of toxin caused complete displacement of receptor bound I from the membranes.

When less than a stoichiometric amount of label I was added to the membranes, the concentration of free label decreased with time. Calculations showed that the apparent affinity of the receptor for label I increased nearly two-fold during the 30-min period following label addition. A similar increase in affinity was observed when the binding of I was measured by inhibition of α-toxin binding.

Acetylcholinesterase is an extremely important enzyme, since it is responsible for hydrolyzing acetylcholine that diffuses away from the receptor surface. Many drugs and other toxic agents exert their pharmacological effect by inhibiting this enzyme, and so it becomes important to understand the molecular mechanism of such interactions. The interaction of acetylcholine with its nicotinic receptor is the first step in a series of events which ultimately lead to the expression of the pharmacological action of this agonist. The

acetylcholine receptor is also the target site for other nicotinic agonists and antagonists. These studies should help to elucidate the molecular events which follow the occupancy of the acetylcholine receptor by agonist and antagonist drugs.

Spin-labeled analogs of the drugs edrophonium and propidium will be prepared in the hope that these labels will probe the active and peripheral sites respectively. It may also prove possible to use these spin labels to estimate the distance between the active and peripheral sites. The propidium label will also be used to study the acetylcholine receptor, since it is known that propidium itself binds to this protein.

Publications:

Wee, V.W., Sinha, B.K., Taylor, P.W. and Chignell, C.F.: Interaction of spin-labeled bisquaternary ammonium ligands with acetylcholinesterase. Mol.Pharmacol., in press.

Sinha, B.K. and Chignell, C.F.: The synthesis and pharmacology of some spin-labeled analogs of biotin, hexamethonium, decamethonium, dichlorisoproterenol and propranolol. J.Med. Chem. 18: 669-673, 1975.

Chignell, C.F., Starkweather, D.K. and Sinha, B.K.: A spin label study of egg white avidin. J.Biol.Chem. 250: 5622-5630, 1975.

Table 1

Dissociation constants determined from enzymatic, fluorescence or electron spin resonance methods

Analog	I_{50}[a] ($M \times 10^6$)	K_D[b] ($M \times 10^6$)	K_D[c] ($M \times 10^6$)
I	0.25[d]	0.19	0.36
II	0.18[d]	0.12	0.20
III	3.3[d]	2.9	5.20
Decamethonium	5.1	--	5.9[e]

[a] Determined from the inhibition of *Torpedo californica* acetylcholinesterase.
[b] Determined by ESR titration.
[c] Determined by back titration of bis(3-aminopyridinium)-1,10-decane.
[d] Data taken from Sinha and Chignell, J.Med.Chem. 18: 669, 1975.
[e] Data taken from Taylor and Jacobs, Mol.Pharmacol. 10: 93, 1974.

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Table 2

Binding parameters determined from ESR measurements with native and modified acetylcholinesterase

Serine modification[a]	Ligand	Buffer ionic strength[b]	Binding parameters			
			N_1[c]	K_1 (M^{-1} x 10^{-6})	N_2[c]	K_2 (M^{-1} x 10^{-6})
-	I	High	0.8	5.20	3.4	0.02
-	II	High	0.8	8.49	2.4	0.02
-	III	High	1.1	0.34	---	--
-	I	Low	1.3	30[d]	---	--
-	II	Low	1.3	285[d]	---	--
-	III	Low	1.1	8.59	---	--
+	I	High	1.0	0.81	---	--
+	II	High	1.1	0.54	---	--

[a] Enzyme reacted with N-methyl-3-hydroxypyridinium iodide methanesulfonate.
[b] High ionic strength 0.1 M NaCl, 0.04 M $MgCl_2$, 0.01 M Tris-HCl (pH 8.0); low ionic strength 0.001 M NaCl.
[c] Moles of ligand bound per 80,000 dalton subunit.
[d] These values can only be considered as estimates of the inherent inaccuracy of the method.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02509-02 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Acridine Spin Labels as Probes for Nucleic Acids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. Wee	Visiting Fellow	PB NHLI
OTHER:	C. F. Chignell	Pharmacologist	PB NHLI
	B. K. Sinha	Guest Worker	PB NHLI

COOPERATING UNITS (if any)
Dr. B. K. Sinha is affiliated with Microbiological Associates, Bethesda, Maryland 20016

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

SUMMARY OF WORK (200 words or less - underline keywords)

A series of 9-aminoacridine spin labels has been synthesized (see Project No. Z01 HL 02517-01 PB) and their electron spin resonance (ESR) spectra recorded in the presence of DNA, poly dA-dT and poly dG-dC. A comparison of the maximum anisotropic hyperfine splittings ($2A_{\prime\prime}$) for 4-[(6-chloro-2-methoxy-9-acridinyl)-amino]-2,2,6,6-tetramethyl-1-piperidinyloxy (I) and 4-(9-acridylamino)-2,2,6,6-tetramethyl-1-piperidinyloxy (II) bound to DNA, poly dA-dT and poly dG-dC provided direct evidence that II preferred dA-dT base-pairs while I showed no base preference. The methiodide (III) and ethiodide (IV) salts of II also seemed to bind preferentially to dA-dT base-pairs. Polydeoxyribonucleotide complexes with label II all exhibited higher melting temperatures (T_m) than complexes with label I. These results suggest the double helix is stabilized by the interaction of the acridine ring with the phosphate backbone so that the substitution of a bulky group on the ring will decrease binding. The ESR spectra of I and II in RNA showed the existence of two distinct species. Comparison of the ESR spectra of I and II in RNA with those in poly A-poly U, poly G-poly C, poly A, poly U, poly C and poly G indicates that the two species were due to single- and double-stranded complexes in RNA. Hence, these labels may serve as useful tools in studying base-pairs specificity during intercalation as well as useful probes for single- and double-stranded regions on nucleic acids.

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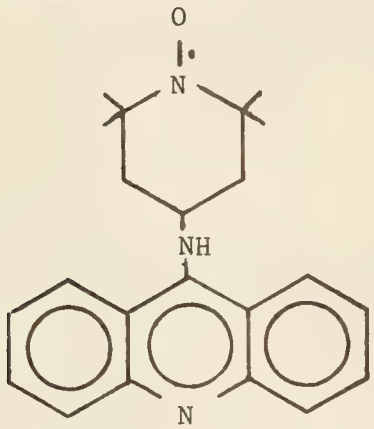
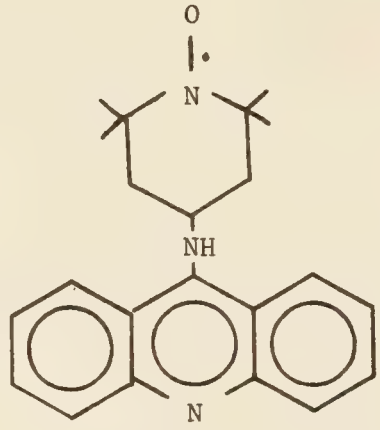
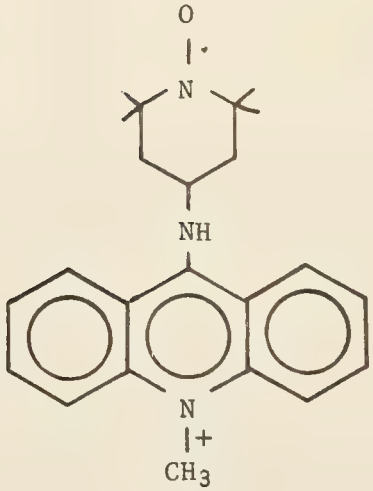
The nature of the interaction between acridine dyes and nucleic acids is of considerable interest not only because of the mutagenic properties of these compounds but also because of their reported anti-tumor activity. It is generally agreed that the biological properties of the acridine dyes arise from their ability to intercalate into nucleic acids or from their close association with the anionic phosphate groups which serve as the backbone of the nucleic acid. We have therefore synthesized a series of 9-aminoacridine spin labels, I, II, III and IV, and have examined their interaction with RNA and DNA by means of electron spin resonance (ESR) spectroscopy. Previous studies have shown that, while spin label I has a greater affinity for DNA (or RNA) than spin label II, it causes a much smaller increase in the melting temperature (T_m) of DNA (Sinha and Chignell, LifeSci. 17: 1829, 1975). These findings suggest that these labels may exhibit different base specificities in their binding to DNA. It is also possible that labels I and II may adopt different conformations on binding to DNA. In the present studies we have employed native DNA as well as different synthetic single- and double-stranded polynucleotides in an attempt to determine the mode of interaction between DNA and labels I and II.



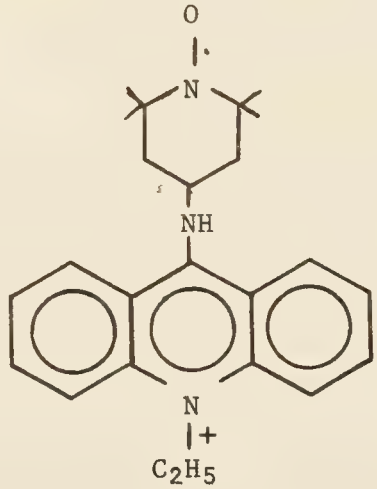
I



II



III



IV

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Electron spin resonance spectra were recorded with a Varian E-4 spectrometer operating at 9.5 GHz. Samples were introduced into the microwave cavity in a flat quartz micro cell. The temperature of the samples was maintained with a Varian variable temperature accessory and was measured with a Yellow Springs Instrument Co. telethermometer.

In the presence of native DNA, poly dG-dC and poly dA-dT, the ESR spectra of spin labels I-IV showed broad asymmetric signals which are characteristic of highly immobilized spin labels. The maximum hyperfine splittings, $2A_{\prime\prime}$ and the T_m values of the complexes are given in Table I. The maximum hyperfine splitting ($2A_{\prime\prime}$) for label I in the presence of poly dA-dT (62.5 G) was greater than that observed in the presence of poly dG-dC (57 G). However, the $2A_{\prime\prime}$ value for DNA ($\sim$ 59 G) was intermediate between those observed for poly dA-dT and poly dG-dC. A closer examination of the high field ESR spectra of I bound to DNA at varying molar ratios clearly showed an initial peak ($2A_{\prime\prime}$ = 62 G) at high DNA/I ratio, which gradually changed into two well-defined peaks at lower DNA/I ratios, finally ending as an intermediate broad peak. These results suggest that there is no base-preference in the intercalation of I into DNA. On the other hand, similar studies with II in DNA clearly showed a $2A_{\prime\prime}$ value equivalent to that observed of II in poly dA-dT. Spin labels III and IV also closely resemble II in their binding to nucleic acids. These findings provide direct evidence for a preferential base-pair intercalation by some analogs of 9-aminoacridines.

Table I

The maximum hyperfine splittings ($2A_{\prime\prime}$) and melting temperatures (T_m) of complexes between the 9-aminoacridine spin labels and various nucleic acids

Nucleic Acid	I		II		III		IV	
	$2A_{\prime\prime}$ (G)	T_m (°C)	$2A_{\prime\prime}$ (G)	T_m (°C)	$2A_{\prime\prime}$ (G)	T_m (°C)	$2A_{\prime\prime}$ (G)	T_m (°C)
Native DNA	59	69	55.5	78	57	$\sim$ 68	56	70
poly dA-dT	62.5	54	55.5	60	57	50	57	56
poly dG-dC	57	72	62.5	> 78	60	> 78	60.5	> 78

Kleinwächter and Koudelka (Biochim.Biophys.Acta 91: 539, 1964) have shown that the binding of acridines to dG-dC base-pairs causes a larger increase in T_m than binding to dA-dT pairs. However, it seems unlikely that the higher T_m value of the DNA-II complex can be attributed to the preferential binding of II to dG-dC base-pairs, since the ESR studies suggest that this label binds

to dA-dT regions in DNA. Furthermore, in the presence of poly dA-dT, II still induces a higher T_m value than I. Possibly the higher T_m value of the DNA-II complex results from stabilization of the DNA double helix by a Coulombic interaction between the acridine nitrogen and anionic phosphate groups on the outside of the nucleic acid. Spin label II, being unsubstituted, offers less steric hindrance to such an interaction. The observation that III and IV have T_m values closer to I than II suggests that quaternization may reduce affinity for the phosphate groups perhaps as a result of steric hindrance.

Since it had been previously reported that II inhibits DNA-dependent RNA polymerase whereas I stimulates, one final phase of this work will be to study the biological activity of III and IV in DNA-dependent RNA polymerase to provide further support for hypothesis.

Previous studies on spin label II bound to calf-liver RNA have shown that there are two different species formed in solution (Sinha and Chignell, Life Sci. 17: 1829, 1975), one of which is characteristic of a highly immobilized label while the other is that of a fairly mobile label. Our present ESR studies of I and II bound to synthetic double-stranded poly A-poly U and poly G-poly C show spectra characteristic of highly immobilized labels, while in the presence of single-stranded poly A, C and U, they show a three-line spectra characteristic of more mobile labels. Therefore, the ESR spectrum of label II complexed to calf-liver RNA is probably a compound spectrum derived from spin label bound to single- and double-stranded regions of the nucleic acid. Since RNA is basically a single-stranded molecule, the only helical sections formed must be due to the folding back of the chain upon itself. RNAs from different sources have been reported to have different helical contents, *eg.* viral RNA = 88% and ribosomal RNA = 78%. We have therefore an ideal tool to probe regions of RNA containing single or double strands.

Acridines are of great interest for two primary reasons: First, because of their biological and pharmacological effects and secondly, because they may be useful tools for probing the conformation of nucleic acids in biologically important complexes such as chromatin. This information may also be usful in determining the different stages which inhibition and stimulation of RNA synthesis takes place during transcription of genetic information.

Publications:

Sinha, B.K. and Chignell, C.F.: Acridine spin labels for probes for nucleic acids. LifeSci. 17: 1829-1836, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02513-04 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of $Na^+ + K^+$-ATPase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	U. Honegger	Visiting Fellow	PB NHLI
OTHER:	T. N. Lo	Visiting Fellow	PB NHLI
	E. O. Titus	Research Chemist	PB NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pulmonary

SECTION

Molecular Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Cyanylation of the sulfhydryl groups of $(Na^+ + K^+)$-ATPase, an enzyme responsible for cation transport in cell membranes, by 5×10^{-3} M 5-thiocyano-2-nitrobenzoic acid, causes approximately 30% inactivation. Na^+ plus ATP protect the enzyme from cyanylation and inactivation, whereas phosphorylation by Na^+ plus ATP plus Mg^{++} enhances cyanylation without affecting inactivation. The data suggest the presence of two populations of sulfhydryl groups in this ATPase.

Cleavage of the major protein of the enzyme occurs at different points depending on the presence or absence of sodium during trypsin action.

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PHS-6040
(12-75)

Project Description:

Objectives: The sodium plus potassium-dependent ATPase is the active transport system that moves monovalent cations across cell membranes against electrical and chemical gradients and thus maintains the excitability of nerve and muscle. It is of interest as the receptor for digitalis glycosides and is a model for the study of information transfer across cell membranes, since extracellular glycosides regulate the intracellular catalytic site. The goals of this project are to describe the molecular events (presumbly reversible changes in conformation of the ATPase) that are associated with ion transport and to ascertain the major structural features of the system.

The enzyme, as isolated from several membranes, is a particulate complex of lipids and two proteins. Our earlier studies have shown that the larger of these proteins traverses the whole membrane and undergoes major conformational changes as the transport enzyme is phosphorylated and dephosphorylated during ion movement. Certain of these conformational changes protect and others expose protein sulfhydryl groups to reaction with radioactive labeling reagents such as N-ethylmaleimide. Current research is directed toward the location of these conformationally sensitive sites as a first step in structural studies of the enzyme.

Methods Employed: Certain of the conformational states through which the enzyme must pass during ion transport are stabilized by adding ionic ligands, or by ion-induced phosphorylation with ATP. Sulfhydryl groups are labeled by ^{3}H or ^{14}C-N-ethylmaleimide. The distribution of label in peptide fragments obtained by partial proteolysis of the labeled protein is then compared with that obtained from control enzyme labeled in the ground state without ligands.

Conformationally altered and control enzymes are also labeled by cyanylation of cysteine sulfhydryl groups with 5-thio[^{14}C]-cyano-2-nitrobenzoic acid (TCNB). In theory (Degani and Patchornik, Biochemistry 13: 1, 1974), the resulting cyano-proteins should be cleavable by mild alkali at the cyanylation sites providing a competing base-catalyzed decyanylation can be minimized. Conformationally dependent changes in cleavage sites should be revealed by the molecular weights of the resultant peptide fragments.

Purified ATPase is prepared from rabbit kidney microsomal fragments by modification of Jørgensen's (Methods in Enzymology 32: 277, 1974) procedure. Conventional analytical disc gel electrophoresis of membrane proteins solubilized in sodium dodecyl sulfate is used to characterize ATPase and its hydrolytic products.

Major Findings: Although the minor protein of ATPase is resistant to trypsin, the major, conformationally active protein of molecular weight 100,000, is cleaved near the center, since fragments of approximately 40,000 and 50 to 60,000 molecular weight can be identified. In the presence of sodium, cleavage is initiated near one end, since the first major fragment

has molecular weight of approximately 80,000.

Kinetic studies of the inactivation of ATPase (2 mg/ml) by 5 mM TCNB at 37° were being initiated at the time of the last report. Rapid inactivation of about 30% of the enzymatic activity is complete in 2 to 4 minutes and is followed by a slow inactivation caused solely by exposure to 37° temperature. About 50% of the TCNB-dependent inactivation can be prevented by inclusion of the substrates, 150 mM NaCl, and 50 mM ATP, but under phosphorylating conditions (Na^+ plus ATP plus 10 mM Mg^{++}) no change in the rate of inactivation is observable. ^{14}C-TCNB has been synthesized and the rate of cyanylation of ATP has been measured by isotope incorporation into particles retainable by Millipore filters. Na^+ plus ATP protects the enzyme against ^{14}C incorporation, but phosphorylation with Na^+ plus ATP plus Mg^{++} markedly enhances isotopic labeling. The combined enzymatic and radioactive measurements thus suggest the presence of at least two populations of sulfhydryl groups in ATPase. Both of these would be sensitive to ligands, but only one would be in a position to moderate enzymatic activity.

^{13}C-TCNB has been synthesized for future use in nuclear magnetic resonance investigations.

Significance to Biomedical Research: Characterization of the ion transport system is essential to an understanding of the regulatory mechanisms of the cardiovascular system. Other ion transport systems, most notably that for calcium in sarcoplasmic reticulum, strongly resemble the ATPase in amino acid distribution, molecular weight, sensitivity to SH reagents and the intermediary role of a high energy glutamyl-phosphate moiety. It is to be hoped that similar relationships between structure, amino acid sequence and the ability to couple useful conformational alterations to metabolic energy will emerge in several transport systems.

Proposed Course of Project: Further work on the effect of conformational changes on cleavage by trypsin and other hydrolytic enzymes will be necessary if these procedures are to be used for structural information. Studies of the cleavage patterns of cyanylated ATPase will be initiated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02514-02 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Chemical Characterization of Pharmacological Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. O. Titus	Head, Sect. on Mol. Pharmacol.	PB NHLI
	U. Honegger	Visiting Fellow	PB NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

N-Arylethyl-maleimides that can be coupled with ^{35}S and ^{3}H-labeled sulfanilic acid to form radioactively labeled sulfhydryl reagents have been synthesized and will be used to study the effects of agonist-dependent conformational changes on the reactivity of protein SH groups of adrenergic receptors in pineal bodies and smooth muscle. In an effort to provide material sufficiently enriched in receptor content to make such studies practical, β-adrenergic receptors of pineal bodies have been fractionated by differential centrifugation and salt extraction.

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Project Description:

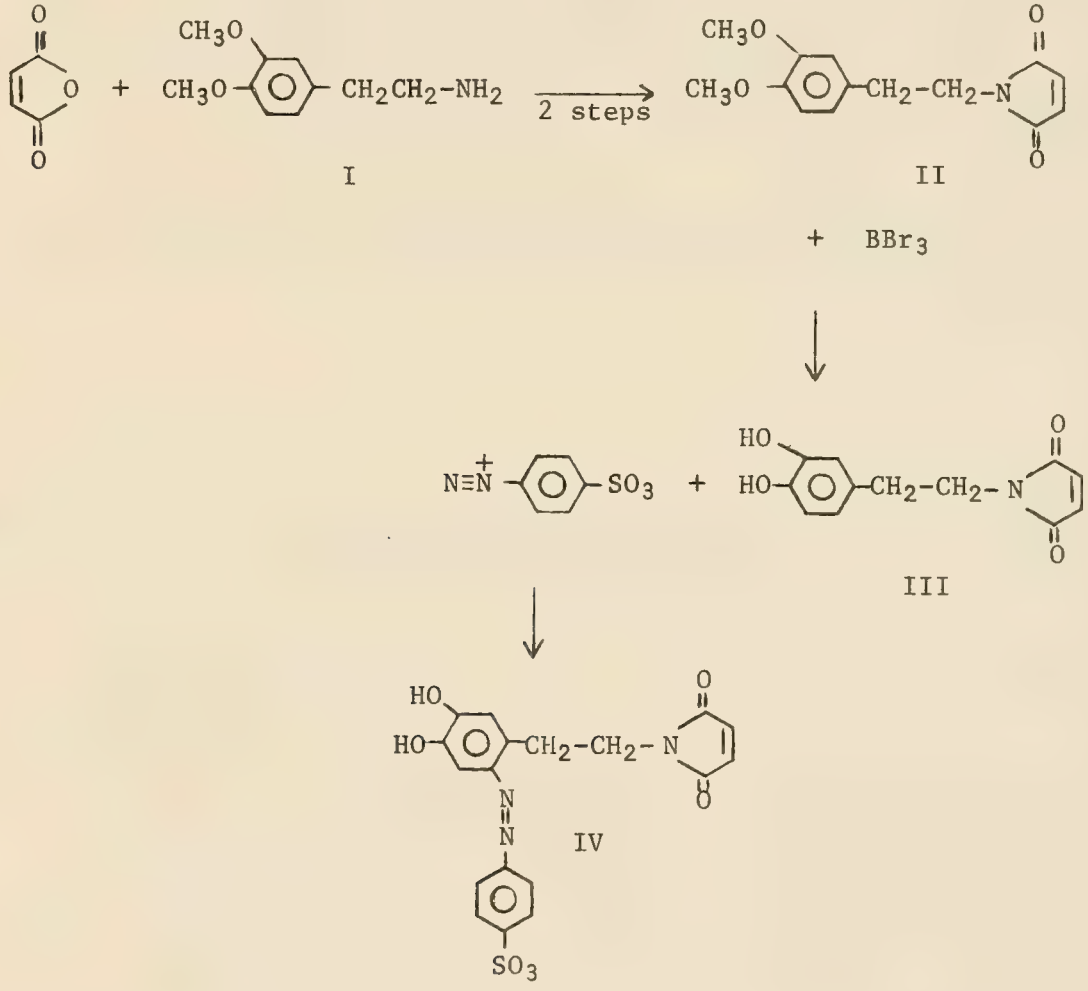
Objectives: The molecular mechanisms by which neurohumoral transmitters activate receptors in mammalian smooth muscle, secretory glands, etc., are unknown. The first step is almost certain adsorption to a recognition site which exhibits high affinity for a limited range of organic structures related to the natural agonist. Conformational changes associated with this adsorption initiate the response. The goal of this project is to isolate those components of adrenergic and other receptors which serve as agonist-recognition sites in smooth muscle and other autonomically innervated mammalian tissues.

Studies of the nicotinic cholinergic receptor isolated from the electroplax of electric fish together with studies of insulin, angiotensin and other receptors from mammalian tissues indicate that individual receptors are characterized by specific proteins. These may be oligomeric assemblies of subunits, among which are the proteins bearing the recognition sites. The introduction of site-directed, covalently bound radioactive labels into such proteins should be useful in isolating receptors. Although the specific agonist-binding properties of the electroplax receptor survive solubilization in detergents and separation from the membrane matrix, studies with the mammalian muscarinic receptor indicate that this may not always be the case. Even if it is impossible to purify a receptor without denaturation, the isolation of a labeled polypeptide, unequivocally identifiable as derived from the receptor, is of interest, since the information which defines the receptor is inherent in the amino acid sequence of the protein.

Methods Employed: The fractionation of membrane proteins is followed by measuring their capacity to bind selectively the physiologically active stereoisomers of radioactively labeled agonists and antagonists. Fractionations are also followed by radioactive labels covalently attached to SH, COOH, or tyrosyl moieties since, with appropriate choice of reagents, these functional groups can be labeled in intact tissue under physiological conditions. Since the adsorption of an agonist causes conformational perturbation of the receptor substance and since conformational change is frequently manifested in altered reactivity of the above-mentioned moieties, ligand-dependent changes in labeling patterns are used for the identification of receptor-related proteins. The procedure is best controlled by a double isotope procedure in which identical preparations are treated with the same reagent, that reagent being labeled with different isotopes as it is used in the presence or the absence of the agonist which alters the conformation. The two preparations are mixed and the individual proteins are isolated from membranous subfractionations. Changes in the ratio of the isotopes identify those proteins affected by the agonist.

Major Findings: Double isotope procedures applicable to the minute quantities of receptors in mammalian tissues require radioactive labels with specific activities of the order of Curies per mole. Both ^{35}S and ^{3}H-labeled sulfanilic acid are now available (see previous annual reports) for diazotization and coupling to protein SH, tyrosyl and imidazole groups. Since

diazotized sulfanilic acid can also react with some of the agonists used to protect receptors, reagents more specific for SH groups are being synthesized by coupling the labeled sulfanilic acids to N-aryl-maleimides. Monohydric phenolic derivatives have been synthesized but were insufficiently reactive with diazo-compounds. Catechol derivatives, although less stable, offer the dual advantages of structural similarity to naturally occurring agonists and enhanced reactivity with the labeled diazo-compounds. Synthesis of a reagent for membrane sulfhydryl groups is being attempted by the following reactions.



Synthesis of the ring-closed intermediates II and III has been completed.

A procedure for the identification of β-adrenergic receptors by the binding of the inhibitor, ℓ-^{3}H-alprenolol (Mukherjee et al., J.Biol.Chem. 250: 4869, 1975) has been modified for use with rabbit pineal bodies, which are commercially available potential sources for receptor isolation. Of the total receptors 26, 30 and 44% are found in membrane, mitochondrial and microsomal fractions respectively when pineal homogenates are centrifuged. In efforts to separate non-receptor proteins from receptor bearing fragments by

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extraction with increasing amounts of ionic detergents, inactivation of receptors has occurred more rapidly than solubilization of protein. Salt extractions, however, have given 50% enhancement of specific activity and a systematic study of the effects of lyotropic salts on receptor activation and solubilization is under way.

Identification of those molecules which bind hormones and transmitters at the receptor sites is important to an understanding of physiological regulatory mechanisms. Autoimmune responses to protein components of receptors have already been implicated in some disease states. The selective action of the β-2-sympathomimetic drugs for bronchial smooth muscle receptors is ascribed to structural features of these receptors which have not yet been identified. Efforts at purification of adrenergic receptors will continue. Should these be successful, application of the methods to α-adrenergic receptors and studies of the physiological effects of antibodies to receptor components will be undertaken.

Publications:

Titus, E.O.: Characterization of pharmacological receptors. Naunyn-Schmiedeberg'sArch.Pharmacol. 288: 269-281, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02517-01 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Synthesis and Biological Properties of some Spin-Labeled 9-Aminoacridines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. F. Chignell	Pharmacologist	PB NHLI
OTHER:	B. K. Sinha	Guest Worker	PB NHLI
	R. L. Cysyk	Pharmacologist	LCP NCI

COOPERATING UNITS (if any)
Dr. R. L. Cysyk, Laboratory of Chemical Pharmacology, National Cancer Institute; Dr. D. B. Millar, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland 20014

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

Five spin-labeled 9-aminoacridines, each bearing either a 4-(2,2,6,6-tetramethyl-1-piperidinyloxy) or a 3-(2,2,5,5-tetramethyl-1-pyrrolidinyloxy) moiety in the 9-position, have been synthesized and assayed for biological activity in three different test systems. Sedimentation velocity measurements indicated that the labels caused unwinding of calf thymus DNA. Those acridines which contained both 6-chloro and 2-methoxy substituents were less toxic to leukemia L1210 cells in static culture than the corresponding unsubstituted analogs. While the unsubstituted aminoacridines were quite good inhibitors of *E. coli* DNA-primed RNA polymerase, the 6-chloro-2-methoxy substituted compounds stimulated this enzyme system. In the presence of *E. coli* DNA, the electron spin resonance spectrum of 4-[6-chloro-2-methoxy-9-acidinyl)amino]-2,2,6,6-tetramethyl-1-piperidinyloxy became broad and asymmetric with a maximal hyperfine splitting ($2T_{\parallel}$) of 57.5 gauss. This finding indicates that when the acridine spin label intercalates into DNA the piperidinyl moiety that bears the nitroxide group becomes highly immobilized. These results suggest that the spin-labeled 9-aminoacridines will be useful probes for nucleic acids.

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(12-75)

The biological properties of the 9-aminoacridines and related analogs are believed to result from their intercalation into DNA (Peacocke, A.R., in Acheson, R.H. (Ed.): Acridine. John Wiley and Sons, New York, 1970, p. 723). Evidence for the intercalation mechanism has come from a wide variety of techniques including viscometry and sedimentation measurements (Lerman, J.Mol. Biol. 3: 18, 1961), electron microscopy (Freifelder, J.Mol.Biol. 60: 401, 1971) and X-ray crystallography (Tsai et al., Proc.Nat.Acad.Sci.U.S.A. 72: 628, 1975). Only two previous reports have described the use of electron spin resonance (ESR) to monitor the interaction of planar ligands with DNA. Ohnishi and McConnell have studied the intercalation of the chlorpromazine cation radical into DNA (J.Amer.Chem.Soc. 87: 2293, 1965) while Ishizu and coworkers (Biochemistry 8: 1238, 1969) have examined the binding of the 5-methylphenazinium cation radical to nucleic acids. The instability of these cation radicals together with their highly complex ESR make these ligands unsuitable as probes for nucleic acids. By contrast, acridines containing the stable nitroxide radical should be useful as probes not only for nucleic acids but also for biologically important complexes containing DNA, *eg.* chromatin. We have therefore synthesized six analogs of 9-aminoacridine (Table 1) and have assayed their biological activity by three different procedures: (a) their ability to cause the unwinding of calf thymus DNA (Table 2); (b) their toxicity to L1210 cells in static culture (Table 3); (c) their effect on *E. coli* RNA polymerase *in vitro* (Table 4).

The acridine spin labels (Table 1) were synthesized by direct condensation of the corresponding aliphatic amines with 9-chloroacridine in the presence of methanesulfonic acid. Since the nitroxide radical is unstable in strongly acidic solutions, an excess of spin label was employed while the methanesulfonic acid was present in only catalytic amounts. ESR measurements were made with a Varian E-4 spectrometer operating at 9.5 GHz.

Each of the spin-labeled acridines demonstrated some biological activity in all three test systems. The increase in the sedimentation coefficient of DNA caused by the spin labels (Table 2) strongly suggests that these compounds intercalate into the nucleic acid. Intercalative binding was also indicated by the ESR measurements which clearly showed (Sinha and Chignell, LifeSci. 7: 1829, 1975) that the nitroxide-bearing heterocyclic rings of labels I, II, IV-VI became highly immobilized in the presence of DNA.

All of the spin labels exhibited cytotoxicity when tested against leukemia L1210 cells in static culture. The results are shown in Table 3 together with the cytotoxicity data for VII, a 9-aminoacridine analog synthesized by Cain and co-workers (J.Med.Chem. 17: 222, 1974) which is currently undergoing preclinical trial. While all of the spin-labeled acridines were toxic after both 24 hr and 48 hr of incubation, labels IV and VI were considerably more effective after the 48-hr period. The lower toxicity of labels IV and VI at 24 hr may be due to slower transport to binding sites on nuclear DNA.

While labels I-III are inhibitors of *E. coli* DNA-primed RNA polymerase, they are less active than 9-aminoacridine itself (Table 4). By contrast, labels IV-VI exhibited a dose-dependent stimulation of RNA polymerase. Although the

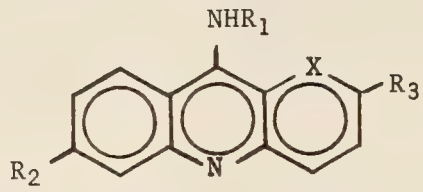
stimulation of RNA polymerase may at first appear surprising, it should be pointed out that O'Brien and co-workers (Proc.Nat.Acad.Sci.U.S.A. 55: 1511, 1966) and Krakow (Biochim.Biophys.Acta 72: 566, 1963) have reported similar results for spermine and other polyamines. Krakow has suggested that this stimulation may result from binding of the polyamines to RNA which may in turn relieve product inhibition of the polymerase.

These results show that the spin-labeled acridines do bind to nucleic acids and that they exhibit many of the properties of the parent 9-aminoacridine. In future studies, these spin labels will be used in ESR studies to probe DNA, RNA and biologically important complexes between nucleic acid and proteins such as chromatin.

Publications: Sinha, B.K., Cysyk, R.L., Millar, D.B. and Chignell, C.F.: Synthesis and biological properties of some spin-labeled 9-aminoacridines. J.Med.Chem., in press.

Table 1

The Structures of the Acridine Spin Labels



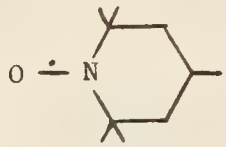
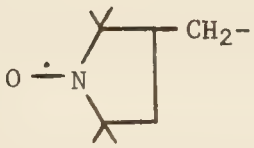
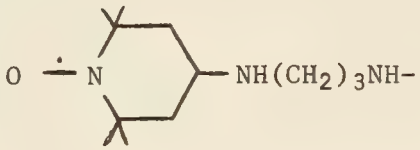
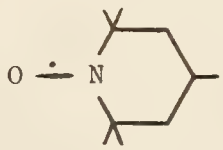
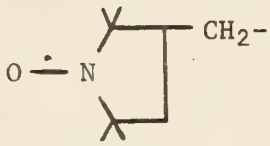
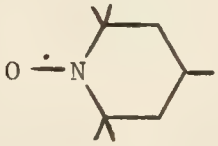
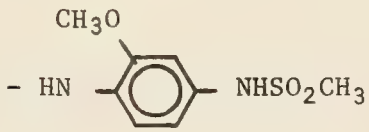
Label	R_1	R_2	R_3	X
I		H	H	C-H
II		H	H	C-H
III		H	H	C-H
IV		Cl	OCH_3	C-H
V		Cl	OCH_3	C-H
VI		Cl	OCH_3	N
VII		H	H	C-H

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Table 2

Sedimentation Coefficients for DNA-Acridine Spin Label Complexes

Drug	Drug Concentration ($M \times 10^6$)	DNA Concentration (µg/ml)	S_{20} DNA	S_{20} DNA + drug	Ratio $\frac{S_{20} \text{ DNA}}{S_{20} \text{ DNA + drug}}$
I	5.2	35	14.3	13.7	1.04
II	5.2	35	14.3	13.2	1.08
IV	5.2	35	13.7	13.4	1.02
V	5.2	35	14.4	13.9	1.04
VI	5.2	35	14.4	13.7	1.05
Proflavine	20	33	15.4[a]	13.5[a]	1.13[a]

[a]Data taken from Lerman (J.Mol.Biol. 3: 18, 1961).

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Table 3

Cytotoxicity of the Acridine Spin Labels Against L1210 Leukemia Cells *in vitro*

Drug Concentration (µg/ml)	% Inhibition of Growth[a]													
	I		II		III		IV		V		VI		VII	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
100	53	87	48	82	47	86	6	78	40	74	0	38	57	92
10	49	84	46	79	56	87	11	63	35	76	0	15	50	87
1	27	56	12	29	10	16	9	24	6	19	0	0	42	82
0.1	1	7	3	2	0	0	12	7	0	5	0	0	29	75
0.01	0	2	0	3	0	0	3	8	10	7	0	0	6	8

[a]Cytotoxicity was determined *in vitro* using leukemia L1210 cells (10^5 cells/ml) in a static culture according to the procedure of Moore et al. (J.Nat.CancerInst. 36, 1966).

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Table 4

Effect of the Acridine Spin Labels on *E. coli* DNA-primed RNA Polymerase

Drug Concentration (µg/ml)	RNA-Polymerase Activity[a] (% control)						
	I	II	III	IV	V	VI	9-amino-acridine
400	9	10	12	116	107	107.5	1.8
200	21	22	20	125	102.5	114	4.0
100	36	45	31	129	106	110	31.0
50	77	70	42	120	106	110	31.0
25	96	87	71	108	112	108	40.0

[a]Assayed by the procedure of Bollum (Procedures for Nucleic Acid Research, Vol. II, p. 296, 1971).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02518-01 PB

PERIOD COVERED
November 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Factors Affecting the Binding of Drugs to Plasma Albumin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. W. Boobis	Guest Worker	PB NHLI
OTHER:	C. F. Chignell	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)

S. W. Boobis is supported by a fellowship from Hoffmann-La Roche Inc. Nutley, New Jersey 07110

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	0.8

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of various factors including disease and the concentration of protein on the binding of drugs to plasma albumin has been investigated. The binding of drugs to albumin from patients suffering from renal failure or hepatic disease and from neonates was reduced. Studies with phenylbutazone showed that there were reductions in both the number and affinity of binding sites on albumin isolated from patients with either renal failure or hepatic disease. Other studies with phenylbutazone, sulfadiazine and salicylic acid indicated that both the number of binding sites and their affinity decreased as the concentration of albumin increased. Circular dichroism and fluorescence polarization measurements suggested that these results were due to a concentration-dependent association (dimerization) of albumin.

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The binding of drugs to albumin provides a useful and versatile model for the study of ligand-macromolecule interactions. An understanding of small molecule-macromolecule association should give an insight into the control of cellular activity and the way it is influenced in health and disease by drugs and other foreign compounds. Drug-albumin binding is also an important determinant of the onset, duration and intensity of drug action, and therefore the investigation of binding has immediate practical application in drug research and therapeutics.

Drug binding is reduced in certain disease states (Reidenberg and Affrione, Ann.N.Y.Acad.Sci. 226: 115, 1973) and in neonates (Krasner et al., Ann.N.Y. Acad.Sci. 226: 101, 1973). We have investigated binding of phenylbutazone, salicylic acid and sulfadiazine to albumin isolated from normal subjects, patients and neonates, in an attempt to clarify the causes of reducing binding and to assess the effect of reduced binding on drug distribution and elimination.

Shen and Gibaldi (J.Pharm.Sci. 63: 1698, 1974) have observed that the capacity of albumin for certain drugs apparently increases with decreasing protein concentration. We have confirmed these findings and have put forward possible explanations for this anomalous binding behavior.

Plasma samples were obtained from healthy volunteers, patients with renal and hepatic disease and from newborn infants. Albumin was isolated using the affinity chromatography method of Travis and Pannell (Clin.Chim.Acta 49: 49, 1973). Binding was studied by ultrafiltration, using the Amicon MMC unit, or by the continuous ultrafiltration technique of Campion and Olsen (J.Pharm. Sci. 63: 249, 1974). Circular dichroism measurements of albumin solutions were made at 27° in a Cary 60 spectropolarimeter. Fluorescence polarization studies of albumin were carried out in an Aminco-Bowman spectrophotofluorometer,

Drug binding to albumin from renal and hepatic disease patients and from neonates was reduced. Quantitative studies of binding of phenylbutazone to albumin showed differences between normal albumin and albumin isolated from patient plasma in both the number and the affinities of binding sites. This suggests an alteration in the conformation of the albumin molecule, either because of differences in the primary amino acid structure or because of a strongly bound inhibitor which is not removed by dialysis.

Circular dichroism profiles of dilute and concentrated albumin solutions showed that upon dilution the proportions of alpha-helix and beta-pleated sheet conformation increased. Fluorescence polarization studies of complexes between dansylglycine and albumin revealed increased molecular mobility in dilute albumin solutions, suggesting that albumin may exist in a polymeric form in concentrated solution. It is possible that the reduced affinity of albumin in concentrated solution for drugs may result from a concentration-dependent association of the protein.

We propose to analyze amino acid composition of albumin samples from volunteers and patients to check for possible variations. We also plan to use the

phenylbutazone binding parameters obtained to predict alterations in drug distribution and elimination in patients with reduced binding. Experiments are also in progress to characterize the polymeric form of albumin from concentrated solutions by means of ultracentrifugation.

Publications:

Chignell, C.F.: The Fluorescence of Drug-Protein Interactions. In Chen, R.F. and Edelhoch, H. (Eds.): Concepts in Biochemical Fluorescence Spectroscopy. New York, Marcel Dekker, 1976, pp. 683-709.

Chignell, C.F.: Ligand Binding to Plasma Albumin. In Sober, H.A. (Ed.): Handbook of Biochemistry. Cleveland, The Chemical Rubber Company, in press.

Chignell, C.F.: Protein Binding. In Garrett, E.R. and Hirtz, J. (Eds.): Methods in Drug Metabolism Research. New York, Marcel Dekker, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02519-01 PB

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Spin Label Studies of Erythrocyte Carbonic Anhydrase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. F. Chignell	Pharmacologist	PB NHLI
OTHER:	V. Wee	Visiting Fellow	PB NHLI
	R. J. Feldmann	Computer Specialist	CCB DCRT

COOPERATING UNITS (if any)

Dr. R. J. Feldmann, Computer Center Branch, Division of Computer Research and Technology, NIH; Dr. R. J. Tanis, Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48104

LAB/BRANCH

Pulmonary

SECTION

Molecular Pharmacology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

The topography of the active sites of rhesus carbonic anhydrases (CA) I and II, rabbit CA I, sheep CA II, and dog CA I has been studied with the aid of spin-labeled analogs of acetazolamide and sulfanilamide. Electron spin resonance measurements indicated that the pyrrolidine ring of 2,2,5,5-tetramethyl-3-[(*p*-sulfamoylphenyl)carbamoyl]-1-pyrrolidinyloxyl became highly immobilized when this label bound to the active site of rhesus CA I. As the chain length between the aromatic and pyrrolidine rings was increased, the mobility of the nitroxide group of the enzyme-bound inhibitor progressively increased until with 2,2,5,5-tetramethyl-3-({[(*p*-sulfamoylphenyl)carbamoyl]-methyl}carbamoyl)-1-pyrrolidinyloxyl there was only minimal interaction between the heterocyclic ring and the active site of rhesus CA I. These findings suggest that the active site of rhesus CA I is a cleft about 14 Å deep. Similar experiments indicated that the active sites of rhesus CA II, dog CA I and sheep CA II were similar in size to that of rhesus CA I, while the active site of rabbit CA was somewhat deeper. Spin-labeled inhibitor 2,2,6,6-tetramethyl-4-(*p*-sulfamoylbenzamide) piperidinooxyl became highly immobilized on binding to type II (high activity) CA but exhibited isotropic motion at the active sites of type I (low activity) isozymes. An attempt has been made to explain these results in terms of the three-dimensional structure of the active sites of human CA I and II.

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Erythrocyte carbonic anhydrase (CA) (carbonate hydro-lyase, EC 4.2.1.1) is a zinc-containing metalloenzyme that reversibly catalyzes the hydration of CO_2 (Maren, Physiol.Rev. 47: 595, 1967). This enzyme is specifically inhibited by aromatic sulfonamides that have the general formula $ArSO_2NH_2$, where Ar is either homocyclic or heterocyclic. X-ray crystallographic studies by Kannan and co-workers (NatureNew Biol. 235: 131, 1972; Proc.Nat.Acad.Sci.U.S.A. 72: 51, 1975) have shown that the active sites of human erythrocyte CA I and II consist of a conical cavity at the bottom of which is a single zinc atom. When sulfonamide inhibitors bind to human CA II, the sulfonamide nitrogen (or oxygen) is directly coordinated to the zinc atom while the aromatic portion of the molecule probably interacts with hydrophobic amino acid residues at the active site.

In previous studies we have shown that sulfonamide inhibitors bearing the nitroxide radical (Table 1) can be used as probes to study the active site of erythrocyte CA (Chignell et al., Biochim.Biophys.Acta 271: 6, 1972; Erlich et al., Mol.Pharmacol. 9: 61, 1973). These experiments have demonstrated that the active site of human CA II is about 14 Å in depth. In the present study these same probes have been used to examine the active site topography of rhesus CA I and II, dog CA I, rabbit CA I and sheep CA II.

The ESR spectra parameters of labels I-VI bound to the different isozymes are shown in Table 2. Spin labels I and II became highly immobilized when bound to the active sites of the isozymes. By contrast, label III exhibited anisotropic motion when bound to all of the isozymes with the exception of rabbit CA I. When the chain between the aromatic ring and the pyrrolidine ring was further lengthened to give label IV, an increase in molecular motion was observed for each of the isozymes (Table 2). Spin label V was more mobile than label IV when bound to rabbit CA I but exhibited about the same mobility as label IV at the active sites of rhesus CA II, dog CA I and rabbit CA I. The gradual increase in mobility observed for labels I-VI suggests that the active sites of these CA have a conical shape similar to that of the human isozymes. Labels IV-VI exhibited about the same molecular motion at the active sites of rhesus CA I and II and dog CA I. If the assumption is made that label IV binds to these isozymes in an extended conformation, then it would appear that their active sites are about 13 Å in depth. The observation that maximal mobility is reached with label V for sheep CA II and with label VI for rabbit CA I would indicate that the active sites of these isozymes are somewhat deeper.

Spin label VII was highly immobilized at the active sites of type II isozymes but exhibited isotropic motion at the active sites of the type I CA (Table 3). An examination of the crystal structure of human CA I and II suggests that the substitution of cysteine 204 in isozyme II by serine 204 in isozyme I may be responsible for this observed difference in the mobility of spin label VII. However, sheep CA II also has a serine at position 204 and yet spin label VII is highly immobilized at the active site of this isozyme. The most likely structural difference between human isozymes I and II which could possibly explain the lower mobility of label VII at the active site of isozymes II is presence of phenylalanine 129, which replaces leucine 129 in human CA I. This substitution places an aromatic ring system at a position in the active site

of human CA II where it could interact hydrophobically with the piperidine ring of spin label VII. Recently, Maren and co-workers (Science 191: 469, 1976) have reported that the physiological concentration of chloride ion in the red cell is high enough to cause almost complete inhibition of the carbonate hydro-lyase activity of human CA I. They suggest that carbon dioxide and the bicarbonate ion are not the normal substrates of CA I. The greater mobility of spin label I at the active site of human CA I may therefore reflect a difference in the substrate specificity of this isozyme.

These studies demonstrate the usefulness of spin-labeled sulfonamide for probing the topography of the active site of carbonic anhydrase. The spin-labeled sulfonamides will be used to examine the active sites of other carbonic anhydrases as they become available.

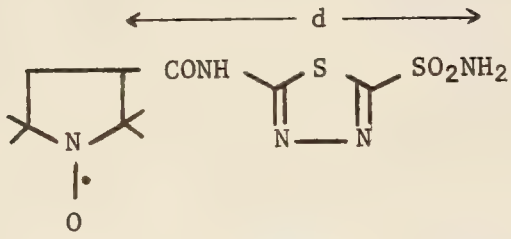
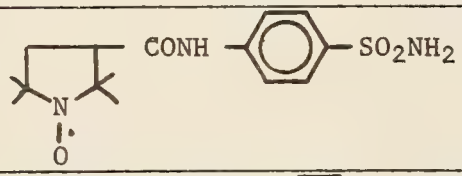
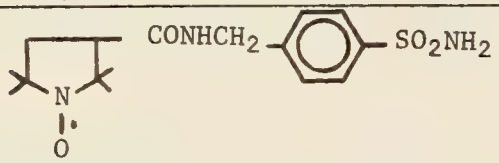
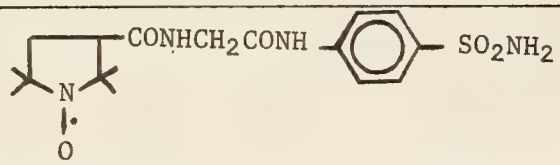
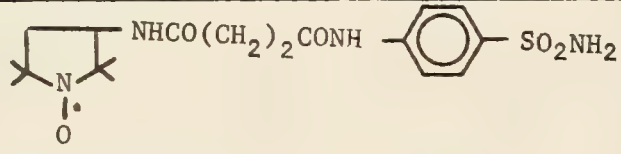
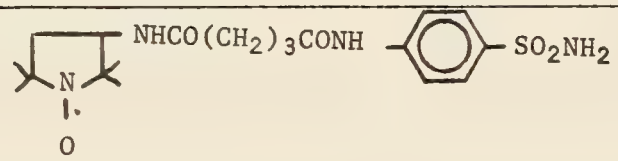
The reversible hydration of CO_2 catalyzed by CA plays an important role in maintaining the acid-base balance of the body. Aromatic sulfonamides, *eg.* acetazolamide, are used clinically as diuretics, and it is thought that their mode of action involves inhibition of CA. It is therefore important to know how these drugs interact with their target enzyme.

Publications:

Wee, V., Feldmann, R.J., Tanis, R.J. and Chignell, C.F.: A comparative study of erythrocyte carbonic anhydrases employing spin-labeled analogs of inhibitory sulfonamides. Mol. Pharmacol., in press.

Table 1

The structures of the Sulfonamide spin labels

	Structure	d (Å)
I		7.44
II		8.16
III		8.88
IV		11.28
V		12.72
VI		14.72
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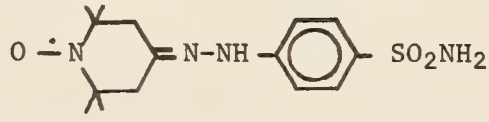
VIII		--

1007

Table 2

The ESR spectral parameters of the sulfonamide spin labels bound to carbonic anhydrases from various mammalian species

Spin label	d^a (Å)	Carbonic anhydrase									
		Rhesus I		Rhesus II		Dog I		Rabbit I		Sheep II	
		τ_c (nsec)	$2T_{\prime\prime}$ (gauss)	τ_c (nsec)	$2T_{\prime\prime}$ (gauss)	τ_c (nsec)	$2T_{\prime\prime}$ (gauss)	τ_c (nsec)	$2T_{\prime\prime}$ (gauss)	τ_c (nsec)	$2T_{\prime\prime}$ (gauss)
I	7.44	--	62.0	--	58.0	--	59.5	--	59.0	--	60.5
II	8.16	--	55.0	--	58.5	--	52.5	--	55.0	--	57.5
III	8.88	5.90	--	2.72	--	4.57	--	--	59.0	5.46	--
IV	11.28	2.84	--	1.91	--	2.38	--	3.59	--	4.65	--
V	12.72	3.35	--	1.98	--	2.18	--	3.54	--	2.62	--
VI	14.72	2.35	--	2.88	--	2.54	--	1.91	--	3.56	--

[a] See Table 1.

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Table 3

The ESR spectral parameters of spin label VII bound to erythrocyte carbonic anhydrase from various mammalian species

Carbonic anhydrase	τ_c (nsec)	$2T_{\prime\prime}$ (gauss)
Human I	3.85	--
Human II	--	59.3
Bovine II	--	56.0
Rhesus I	4.35	--
Rhesus II	--	58.5
Dog I	4.41	--
Rabbit I	2.77	--
Sheep II	--	56.5

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02520-01 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Inhibition of Tumor Cell Growth *in vitro* by Anti-inflammatory Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. C. F. de Mello	Visiting Fellow	PB NHLI
	M. A. Beaven	Pharmacologist	PB NHLI
OTHER:	V. Hial	Visiting Fellow	HE NHLI
	Z. Horakova	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)
Dr. V. Hial, Hypertension-Endocrine Branch, National Heart and Lung Institute, Bethesda, Maryland 20014

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Studies with rat hepatoma cell cultures indicate that the nonsteroid anti-inflammatory drugs, in therapeutic and toxic concentrations, reversibly inhibit cell proliferation. A parallel reduction in protein and nucleic acid synthesis is also observed with the exception that, in low concentrations, the salicylate drugs salicylamide, salicylic acid and aspirin stimulate protein and nucleic acid synthesis. In high concentrations, the salicylate drugs inhibit protein and nucleic acid synthesis as well as cell proliferation. The order of potency in inhibiting culture growth, meclofenamate > indomethacin > salicylamide > phenylbutazone > phenacetin > aspirin = salicylic acid, is similar to that reported for their anti-inflammatory activity and their ability to inhibit prostaglandin synthesis. The findings, therefore, may have some bearing on the anti-inflammatory activity of these drugs.

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Project Description

Introduction. In the preceding report (Z01 HL 02502-03 PB), we reported that aspirin and indomethacin in pharmacological doses inhibited growth of two types of transplantable tumors, a mast-cell ascites tumor and Lewis lung carcinoma in mouse. There have been reports by others (Tashjian et al., Prostaglandins 3: 515, 1973; Powles et al., Brit.J.Cancer 28: 316, 1973) that these two drugs reduce size and extent of bone resorbing tumors in animals. The intention of the work described here is to determine if anti-inflammatory drugs, in general, inhibit growth and metabolic activity of tumor cells. The studies were undertaken with rat hepatoma cell (HTC) cultures, since cell proliferation and metabolic activity, as measured by protein and nucleic acid synthesis, could be readily determined in this preparation. The use of cell cultures also enabled a comparison to be made of the relative potencies of the different anti-inflammatory drugs.

Methods employed. Cell cultures were obtained from a line started by E. G. Thompson (Biochem.Biophys.Res.Commun. 56: 605, 1974). The cells were grown in Eagle's medium #2 with 10% fetal calf serum under 95% air and 5% CO_2 at 37°C. Cultures were grown in large disposable screw cap, plastic, flat bottom flasks. Fresh cultures were prepared each week by diluting 1 ml of old culture with 19 ml of fresh medium. For individual experiments suspensions of 4-day-old cultures were diluted with 20 volumes of fresh medium. Five milliliter aliquots of the diluted culture (approximately 100×10^3 cells) were dispensed into 25 ml plastic screw cap culture flasks. The flasks were regassed, drugs were added to cultures either at the start of culture growth (day 0) or on day 4 30 min before the addition of labeled compounds as explained later.

Cell number was determined after removal of Eagle's medium. The cells which form a monolayer on the bottom of the flasks were treated with trypsin and then trypsin soybean inhibitor. The cells were suspended by vortexing and counted by use of a Neubauer counting chamber.

Measurement of protein and nucleic acid synthesis. One microcurie of [3,5-^{3}H]-leucine or [^{3}H]-thymidine or 0.3 µc of [^{14}C]-uridine were added to the cultures. The flasks were regassed and incubated for 3 hr in the case of the labeled leucine and 30 min in the case of the labeled nucleosides. After removal of the medium and washing the cells with Locke's solution, a trichloroacetic acid precipitate of the cells was prepared. The TCA precipitate was washed with ethanol and then dissolved in 200 µl of hyamine hydroxide for assay of radioactivity by liquid scintillation counting. The percent of label incorporated into the TCA precipitable fraction was calculated and compared with that of control cultures (cultures grown in the absence of drug). Values were then calculated as a percent of the mean incorporation observed for control cultures ± SEM.

Findings. Reduction in cell counts in cultures grown in the presence of anti-inflammatory drugs. When added at the start of culture growth, indomethacin and, in higher concentrations, aspirin and sodium salicylate inhibited

cell proliferation as indicated by the reduction in cell count of 4-day-old cultures. The reduction was proportional to drug concentration. The inhibition was reversible; after diluting the cultures to give less than inhibitory concentration of drug, normal growth resumed.

Effect of ^{3}H-leucine incorporation into cell protein. ^{3}H-Leucine incorporation into cell protein was also reduced by the anti-inflammatory drugs. In 4-day-old cultures, a reduction in ^{3}H-leucine incorporation was observed for all of the anti-inflammatory drugs tested. The reduction was proportional to drug concentration, although the slope of the curve was less steep for indomethacin than for other drugs. Meclofenamate and indomethacin were the most potent drugs (50% reduction at 0.03 and 0.1 mM respectively) and phenacetin, aspirin and sodium salicylate the least (50% inhibition 1.5-2.5 mM).

Drugs with little or no anti-inflammatory activity were only weakly inhibitory. *m*-Hydroxybenzoic acid produced 50% inhibition at 9 mM and for complete inhibition of ^{3}H-leucine incorporation of culture growth, 20 mM of the drug was required. The salicylate metabolite sodium gentisate in concentrations of 3 and 5 mM did not inhibit ^{3}H-leucine incorporation.

An unusual feature was that at low concentrations the salicylate drugs, salicylamide, aspirin, sodium salicylate and *m*-hydroxybenzoic acid, stimulated ^{3}H-leucine incorporation without altering cell count, whereas the nonsalicylate drugs, meclofenamate, indomethacin, phenylbutazone and phenacetin, did not stimulate incorporation.

Effect of ^{3}H-thymidine and ^{14}C-uridine incorporation. A reduction in the incorporation of the two labeled nucleosides was observed with cultures grown in the presence of indomethacin. The reduction paralleled that of ^{3}H-leucine incorporation and was proportional to the concentration of drugs. With cultures grown in the presence of low concentration of aspirin, ^{3}H-thymidine, and to a much greater extent ^{14}C-uridine, incorporation was increased. The increase in nucleoside incorporation was apparent even though cell count was significantly diminished, *eg.* 2 mM. In the presence of high concentrations of aspirin, nucleoside incorporation was reduced as were cell count and ^{3}H-leucine incorporation.

Short-term effects of the anti-inflammatory drugs. When drugs were added 30 min before the addition of labeled amino acid or nucleoside, only small decreases in amino acid and nucleoside incorporations were observed, although the stimulation with the salicylate drugs was still apparent. These data suggest that, except for the stimulation observed with salicylates, the anti-inflammatory drugs do not have a marked effect on amino acid or nucleoside incorporation over the short term and that the decreases observed in the earlier studies may be a reflection of decreased cell counts.

Relationship to therapeutic levels, anti-inflammatory activity and ability to inhibit prostaglandin synthetase. The data available on the levels of drugs found in human plasma, or those required to inhibit prostaglandin synthesis or reduce inflammation, are presented in Table 1. The data are

incomplete but permit some general conclusions. Although inhibition of ^{3}H-leucine incorporation and culture growth was observed at levels that can be considered toxic, significant inhibition was observed with high therapeutic levels of indomethacin, salicylic acid and aspirin. Marked inhibition of ^{3}H-leucine incorporation was noted with concentrations of phenylbutazone that were within the normal range of therapeutic plasma levels. Except for salicylamide, drugs can be ranked in the same order of effectiveness in anti-inflammatory activity, the ability to inhibit prostaglandin synthetase and the effect on cell growth, *ie* meclofenamate and indomethacin were more active than phenylbutazone, and sodium salicylate and aspirin were the least active on these systems. *m*-Hydroxybenzoic and gentisic acid were either inactive or inhibitory only at high concentrations. The concentrations of drugs required to inhibit prostaglandin synthetase, however, were much lower than those required to inhibit culture growth (Table 1).

Significance to biomedical research and proposed course of project. The studies indicate that all the common anti-inflammatory drugs inhibit growth and metabolic activity of hepatoma cell cultures. Since their order of potency appears to parallel their efficacy as anti-inflammatory agents and their ability to inhibit prostaglandin synthesis, the findings may have some bearing on the anti-inflammatory properties of these drugs. The findings also suggest that the prostaglandins may in some way be involved in cell replication.

The question of whether or not prostaglandins themselves will reverse the inhibition produced by the anti-inflammatory drugs will be investigated. Other cell cultures will be examined to see if the anti-inflammatory drugs affect cell growth in general. Fibroblast cultures known to produce prostaglandins will be compared with fibroblast lines that are deficient in prostaglandin synthetase. It is hoped ultimately to extend such studies with cells known to be involved with the inflammatory process.

Publications: None

Table 1

Comparison of plasma levels, anti-inflammatory activity and ability to inhibit prostaglandin synthetase and culture growth

	Peak plasma levels in man	Dog spleen prostaglandin synthetase ID_{50}	Rat paw edema ED_{50}	Toxic levels	Inhibition of ^{3}H-leucine incorporation
	(μM)	*(μM)*	*(μmol/kg p.o.)*	*(μM)*	*(μM, range)*
Meclofenamate	7	0.1	50	--	-100
Indomethacin	5-20	0.2	18	--	1-500
Salicylamide	< 4	--	--	--	50-500
Phenylbutazone	260-500	7.2	324	4,200	50-1,000
Phenacetin	--	--	--	--	500-5,000
Salicylic Acid	1,100	--	--	4,000-4,500	500-10,000
Aspirin	320-1,700	37	830	3,000	500-5,000
m-Hydroxybenzoic Acid	--	Inactive <720	Inactive <1,000	--	10,000-20,000
Gentisic Acid	--	Inactive <650	Inactive <970	--	Not inhibitory at 5,000

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02521-01 PB

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Adrenergic and Histamine Receptors in Rabbit Aorta

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Z. Horakova	Pharmacologist	PB NHLI
OTHER:	M. A. Beaven	Pharmacologist	PB NHLI
	E. O. Titus	Head, Sect. on Mol. Pharmacol.	PB NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

SUMMARY OF WORK (200 words or less - underline keywords)

Studies with diazotized sulfanilic acid (DSA) and spirally cut strips of rabbit aorta indicated that this agent reversibly inhibited the contraction induced by norepinephrine but not that by histamine. The data suggested that inhibition was due in large part to inactivation of norepinephrine by DSA but that DSA may have in addition a selective affinity for the adrenergic receptor. Additional studies on rabbit aorta with the H_1 and H_2 histamine antagonists indicated that the response to histamine was mediated by both H_1 and H_2 receptors with H_1 having the predominant effect.

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PHS-6040
(12-75)

Objectives: There were two objectives in the present study. The first was a continuation of previous projects (see Annual Report NHLI-99, 1974) in which the labeling of reactive groups such as tyrosyl, SH, NH and COOH by radioactive derivatizing agents was suggested as a means to identify receptors. In this study the effect of diazotized sulfanilic acid (DSA) on the response of the isolated rabbit aorta to norepinephrine and histamine was studied. The purpose of the study was to determine if this reagent, which has been widely used to label the outward-facing proteins of cell membranes, was inhibitory to those receptors which excite smooth muscle contraction.

The second objective was to investigate the nature of histamine receptors in the aorta. Studies with H_1 and H_2 antagonists in the intact animal indicate that both H_1 and H_2 receptors are responsible for the cardiovascular response to histamine (Parsons and Owen, International Symposium on Histamine H_2-Receptor Antagonists, Deltakos Ltd., London, 1973). In preparations such as the isolated rabbit ear where central connections are severed, the response to histamine also appears to be mediated by both H_1 and H_2 receptors; a pressor effect through H_1 and a depressor effect through H_2 receptors (Ercan and Türker, Naunyn-Schmiedeberg'sArch.Pharmacol. 291: 23, 1975). In this study the possible presence (or absence) of the two types of histamine receptors in a single vessel has been investigated with the rabbit aorta. Previous studies have indicated the presence of only H_1 receptors in this preparation (Fleisch, BloodVessels 11: 193, 1974).

Methods Employed. Spirally cut strips of rabbit aorta were prepared by the methods of Furchgott and Bhadrakom (J.Pharmacol.Exp.Ther. 111: 265, 1954) and Patterson (J.Allergy 29: 165, 1958). The tissue was subjected to various concentrations of DSA for different times. The responses to the different pharmacologic agents were measured before and after treatment with this agent. Dose-response curves were analyzed to determine whether apparent affinity for the drug or the receptor sites was altered.

Findings. Studies with DSA. DSA in concentrations of 2×10^{-6} M to 5×10^{-5} M (final concentration in bath) reduced or in the higher concentrations abolished the response to norepinephrine (10 ng/ml). This reduction was dependent on the dose of DSA. The effect was the same whether the preparation was pre-exposed to DSA for short or long periods (5 to 30 min).

Increasing the amount of norepinephrine partially reversed the DSA-induced blockade. Studies with different concentrations of norepinephrine and DSA suggested that coupling with norepinephrine to form an inactive product was a major factor in the apparent blockade by DSA but did not exclude an additional, easily reversible inhibition of the α-receptor. Normal response to norepinephrine could be restored by washing.

In contrast, the response of the aorta to histamine was not affected by DSA. The preliminary data obtained thus far would be consistent with either a very slow inactivation of histamine by DSA or with a selective affinity between DSA and adrenergic receptors.

Studies with H_1 and H_2 Histamine Antagonists. Histamine in concentrations of 50-200 ng/ml contracted the aorta and the extent of contraction was dependent on dose. This response was completely abolished by H_1 antagonists such as mepyramine (0.1-10 ng/ml). Smaller doses of mepyramine partially blocked the response to histamine. The H_1 antagonist alone had no effect. The H_2 antagonist, burimamide (100-1,000 ng/ml), had the opposite action and enhanced contraction induced by histamine. The enhancement was apparent when the burimamide was added before the histamine or when added after the response to histamine was fully developed. Even threshold doses of histamine were potentiated with burimamide. Burimamide alone produced a slight but perceptible relaxation. Metiamide, another H_2 inhibitor, had a similar effect. Preliminary studies indicated that the effect of H_1 inhibitors was reversed more rapidly by burimamide than by other H_2 inhibitors. These data suggest that the H_1 and H_2 blockers have opposite and competing effects as has been observed with the rabbit ear preparation. Further, the response to histamine appears to be a balance between mepyramine-sensitive H_1 receptors and burimamide-sensitive H_2 receptors with the H_1 effects predominating.

Conclusion and Proposed Course of Project. The preceding studies indicate that the anticipated irreversible formation of adducts with DSA either does not occur with adrenergic receptors in aorta or occurs at sites that do not inhibit adrenergic function. This does not exclude the possibility that conformational changes induced by specific agonstsis or antagonists will alter the labeling by radioactive DSA and this aspect of the problem will be further explored.

Further studies of the H_1 and H_2 receptors in the aorta will be undertaken. The activity of recently synthesized halogenated derivatives of histamine will be investigated in the rabbit aorta.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02522-01 PB

PERIOD COVERED
September 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

A Spin Label Study of Erythrocyte Membranes During the Simulation of Freezing

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. M. A. Nunes	Guest Worker	PB NHLI
OTHER:	Dr. C. F. Chignell	Pharmacologist	PB NHLI

COOPERATING UNITS (if any)

Dr. M. A. Nunes holds a fellowship from the Fundacao de Amparo a Pesquisa de Sao Paulo, Brasil; Dr. Harold T. Meryman, Associate Research Director, Blood Research Laboratory, American National Red Cross, Bethesda, Maryland 20014

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

SUMMARY OF WORK (200 words or less - underline keywords)

This project represents an attempt to understand the mechanism of freeze-induced damage to erythrocyte membranes. Freeze injury was simulated by exposing either red cells or ghosts to hypertonic solutions of sodium chloride from 295-3055 mOsm. Membrane changes were assessed with the aid of spin-labeled probe molecules. Hypertonic sodium chloride solutions produced no detectable changes in electron spin resonance spectra of red cells labeled with stearic acid spin labels. However, an androstane spin label did undergo a small decrease in mobility when red cells were exposed to 3055 mOsm sodium chloride. Ghost membranes labeled with either maleimide or iodoacetamide spin labels showed an increase in the population of highly immobilized labels as the concentration of sodium chloride was increased. These observations suggest that in hyperosmotic sodium chloride an increased rigidity of the red cell membrane develops which is probably due to a decrease in the fluidity of the phospholipids accompanied by conformational changes of the red cell membrane proteins.

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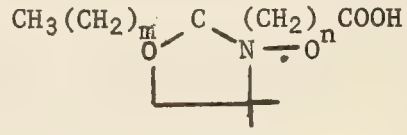
Although the freeze-induced injury of red cells has been extensively studied, the precise molecular mechanism of this phenomenon is still unclear. Meryman has suggested that osmotic stress may be one important factor in the freeze injury of red cells (Cryobiology 8: 489, 1971). We have therefore studied the effect of hypertonic solutions on the fluidity and protein conformation of both red cells and ghosts with the aid of spin-labeled analogs of stearic acid (I,II), androstane (III), maleimide (IV) and iodoacetamide (V).



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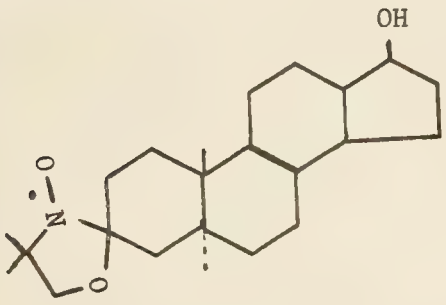
II m = 12, n = 3



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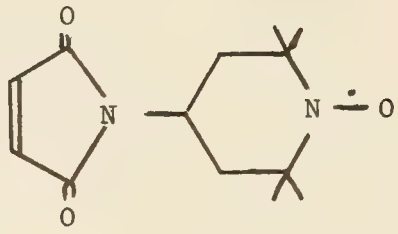

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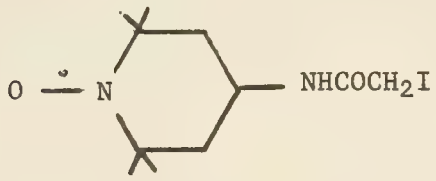

V

For these experiments blood was collected in plastic bags containing a citrate-phosphate-dextrose mixture as an anticoagulant. The blood was stored at 4-6°C and used within five days. Red cells were separated by centrifugation and were washed three times in buffered isotonic NaCl. Red cell ghosts were prepared by the method of Dodge et al. (Arch.Biochem.Biophys. 100: 119, 1963). Electron spin resonance (ESR) measurements were made in a Varian E-4 spectrometer operating at 9.5 GHz. Samples were introduced into the cavity in quartz aqueous sample cells. The mobility of the non-covalently bound labels was expressed either in terms of the order parameter S (II) (Seelig, J.Amer.Chem. Soc. 92: 3881, 1970) or the rotational correlation time τ_c (I and III) (Smith, Biological Applications of Electron Spin Resonance, Swartz, Bolton and Borg, eds., Wiley-Interscience, New York, 1972, p. 483).

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No detectable changes in the mobility of stearic acid labels I and II bound to intact human red cells were observed on exposure to hypertonic solutions of sodium chloride ranging from 295 to 3055 mOsm. However, the androstane label (III) exhibited a 28% increase in τ_c when labeled human red cells were exposed to 3055 mOsm sodium chloride. This finding suggests that there may be a decrease in the fluidity of red cell membrane phospholipids as the osmolality is raised.

Red cell ghosts covalently labeled with N-ethylmaleimide analog IV exhibited ESR spectra which were characteristic of two populations of labels, one of which was more highly immobilized than the other. A decrease of 36% in the ratio of highly immobilized to mobile labels was observed when the solution was raised to 3055 mOsm by the addition of sodium chloride. Red cell ghosts reacted with the iodoacetamide label (V) showed evidence for a single population of fairly mobile labels. The addition of sodium chloride to a final concentration of 3055 mOsm resulted in the appearance of a population of highly immobilized labels.

These observations suggest that in hyperosmotic sodium chloride an increased rigidity of the cell membrane develops, probably due to a decrease in fluidity of the phospholipid moieties accompanied by conformational changes of the red cell membrane proteins. In future experiments we will attempt to determine whether these changes are the result of osmotic gradients or are simply due to an increase in ionic strength. In addition, we will examine directly changes in the membranes of intact red cells that have been frozen with or without cryoprotectants, *eg.* dimethylsulfoxide, glycerol, etc. It is hoped that these studies will provide information on the mechanism of action of these agents.

One of the main problems in blood banking is the storage of intact red cells. While freezing would provide an ideal method of storage, the hemolysis which occurs upon thawing makes this procedure impractical. Although the currently available cryoprotectants reduce hemolysis, none of them provides complete protection. In addition, some cryoprotectants, *eg.* dimethylsulfoxide, have undesirable biological effects which preclude their routine use. The studies which we are undertaking should not only provide a better understanding of the molecular mechanism of freeze-induced injury but may also lead to the discovery of more useful cryoprotective agents.

Publications: Millar, D.B. and Chignell, C.F.: Partial characterization of the 1-anilino-8-naphthalene sulfonate-adrenochrome semicarbazide interaction site in erythrocyte ghost membrane fragments. Biophys.Chem.3: 297-306, 1975.

ANNUAL REPORT OF THE
CLINIC OF SURGERY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

The clinical and laboratory programs of the Surgery Branch have, as in past years, largely centered upon the study of operative methods for the correction of congenital and acquired heart and lung diseases, assessment of the results of such operations, and laboratory studies related to cardiovascular physiology and pharmacology.

Study and Operative Treatment of Hypertrophic Subaortic Stenosis. A major clinical interest of both physicians and surgeons of the Cardiology and Surgery Branches has, since 1960, centered around patients with obstructive cardiomyopathy. Approximately 70 publications have dealt with the clinical, hemodynamic, angiographic, pathologic, and genetic features of this spectrum of diseases. Operative treatment was first carried out for this condition in 1960. In 1975 the results of operative treatment in 85 patients were described. The response to this publication was a striking increase in interest among referring physicians, and over the past year many more operative candidates have been sent to the Institute for evaluation and treatment. As of April 1, 1976 operation has been carried out in 132 patients with IHSS, and 106 patients have been evaluated in detail after operation.

The operative method has been altered and improved. Two vertical incisions are made through the hypertrophied septum beneath the right coronary leaflet of the valve. The bar of muscle between the two incisions is then resected by special knives designed for this purpose. In the total operative experience there have been nine deaths (7%). All surviving patients have derived good symptomatic improvement, and all but two patients were in functional class I or II at the time of most recent examination. Eighty-seven patients had pressure gradients under basal conditions before operation; obstruction at rest was abolished in 72 patients and in the other 15 only trivial pressure differences were recorded. In patients who had residual obstruction early after operation, resolution and eventual disappearance of the pressure gradient has been documented in serial hemodynamic and echocardiographic examinations.

As increasing numbers of patients with IHSS have been seen the disease has been encountered in combination with other serious cardiac malformations. Thus, IHSS has accompanied severe aortic regurgitation, severe mitral regurgitation, discrete congenital subaortic stenosis, and obstructive coronary atherosclerosis. In these patients myotomy and myectomy has been performed along with mitral or aortic valve replacement, resection of subaortic membrane, or aortocoronary bypass grafting. The disease has also been recognized and treated in an increasing number of patients over age 65, and the results of operation in older patients have been as satisfactory as those in younger individuals. Finally, a miniaturized echo transducer has been developed which permits intraoperative measurement of the thickness of

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the interventricular septum. The transducer is mounted on a malleable handle, and is placed in the left ventricular cavity through the retracted aortic valve. Septal thickness measured in this manner has generally correlated well with preoperative estimations of septal thickness. The thickness of the septum has been shown to be 12-14 mm. less immediately after myotomy and myectomy. Further refinement of this instrument will, hopefully, make possible more individualized treatment for patients with this disease.

Protection of the Left Ventricle During Operation. Most cardiac operations require the interruption of coronary blood flow for varying periods. For example, the aorta must be opened for at least one hour in the course of aortic valve replacement. In children with cyanotic forms of congenital heart disease (tetralogy of Fallot, double outlet right ventricle with pulmonic stenosis, total anomalous pulmonary venous connection, etc.) the aorta must be occluded to render the operative field relatively dry for periods of 30 to 60 minutes. The best method for protecting the heart during these periods of ischemia is not known.

Radioactive microspheres were utilized to assess myocardial damage in experiments carried out in dogs. All the animals had hypertrophied hearts, hypertrophy having been induced by the previous creation of large peripheral arteriovenous shunts. When coronary artery perfusion was maintained during bypass (100 min) ventricular function remained good and the ratio of endocardial to epicardial coronary blood flow was increased somewhat one hour after the termination of bypass, i.e. there was no evidence of endocardial ischemia. In other animals coronary flow was not provided, but during bypass (60 min) the heart was rendered deeply hypothermic by continuous external lavage with iced saline solution. These animals showed impairment of left ventricular function after bypass and the endocardial to epicardial flow ratio decreased significantly, clear evidence that persistent subendocardial damage resulted. Experiments have been carried out in which the coronary bed has been perfused with a buffered nutrient solution with high glucose concentration. Preliminary data indicate that, when ventricular function is assessed by function curves, the solution appears to furnish significant myocardial protection.

A Reassessment of Coronary Sinus Ligation. In 1935, Claude S. Beck performed ligation of the coronary sinus as part of an operation he devised to alleviate the symptoms resulting from severe obstructive coronary artery disease. The clinical results with this operation were reportedly good, although it was never widely accepted by other surgeons. Coronary sinus ligation was evaluated by modern methods for the measurement of total and regional myocardial blood flow and for evaluation of left ventricular performance.

The coronary sinus was ligated in ten dogs, and in five others the anterior cardiac veins were ligated as well. Regional myocardial blood flow was determined with radioactive microspheres, and the function of the ventricle was assessed by measurements of filling pressures and LV dp/dt max. When the sinus was ligated the proximal pressure rose from an average of 4 to

37 mm Hg. Total left ventricular blood flow decreased acutely (106 to 87 cc/100 Gm/min) but returned to normal within 30 minutes. Despite this total decrease in left ventricular perfusion, the subendocardial muscle was spared and flow to this area remained essentially unchanged (38 vs. 36 cc/100 Gm/min). This improved subendocardial flow was observed to persist even when total left ventricular blood flow had returned to normal. The performance of the left ventricle, as assessed here, was not altered by coronary sinus ligation. In those animals in which the anterior cardiac veins were ligated also the decrease in total left ventricular blood flow was greater, the subendocardial muscle showed ischemia comparable to that in subepicardial muscle, and one hour after ligation the function of the left ventricle was significantly depressed.

It should be noted that the studies described are of short-term nature. An obvious extension of the observations is to determine the chronic effects of coronary sinus ligation since the procedure could, as Beck believed, be of value in patients with angina pectoris.

Clinical Application and Assessment of Porcine Xenograft Cardiac Valves. Previous reports have described the development in this Institute of the porcine aortic valve, sterilized and preserved in glutaraldehyde and mounted on a flexible stent. This valve, now manufactured by Hancock Laboratories, was first utilized to replace a diseased mitral valve in June 1970. That patient is alive and has normal valve function. Since that time 218 porcine heterografts have been implanted in 177 patients as either isolated or multiple valve replacements. Anticoagulants have not been employed unless the patient had a mechanical (Starr-Edwards or Bjork-Shiley) prosthesis in the aortic position. Four of 80 patients with isolated xenografts have had systemic emboli, two in the immediate operative period and two later. None of the four patients died nor has neurologic disability. Postoperative hemodynamic studies have shown good function of the xenograft in the mitral and tricuspid positions. The valve has not been evaluated in the aortic position for two reasons: the Starr-Edwards aortic valve is relatively satisfactory, and the durability of the xenograft in the aortic position may be less than it is in the atrio-ventricular position.

Because of the excellent hemodynamic function of the xenograft, the extremely low rate of thromboembolism without anticoagulants and its noise-free function this valve has been accepted nationally as the prosthesis of choice for mitral and tricuspid replacement. It is being utilized in the aortic position at several large centers and data concerning its desirability in this application should be available within two years.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02601-02 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Cardiac Valve Replacement with the Hancock Porcine Xenograft: A Six Year Clinical Experience

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI
Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI
Andrew G. Morrow, Chief, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

SUMMARY OF WORK (200 words or less - underline keywords)

Porcine xenograft cardiac valves were first implanted in humans at the NHLI in July 1970. Since then 218 valves have been implanted in 177 patients as either single or multiple replacements. One hundred thirty-five patients have survived operation and have been followed for 3232 months. Early mortality is 12% and late mortality equals 13%. Anticoagulants are administered to patients with either a Starr-Edwards or Bjork-Shiley prosthesis in another position. Four of 80 (5%) patients with isolated xenografts have had emboli; 2 in the early postoperative period and 2 late. Eight of the remaining 97 (8%) mixed valve replacements have had emboli on anticoagulants. Death has not been attributed to thromboembolism in the isolated xenograft group. Hemodynamically the xenograft has been proven satisfactory in any cardiac valve position. Fifteen patients have been followed for periods greater than 4 years and five of these patients for more than five years. Prosthetic dysfunction occurred in one patient 57 months following implant.

1025

Description: Cardiac valve replacement with glutaraldehyde-fixed, flexible stent mounted, porcine xenografts was first performed at the NHLI in July 1970. Since then this prosthesis has gained widespread clinical acceptance and currently is utilized for all atrioventricular valve replacements in this clinic. Hemodynamic, durability, thromboembolic, and patient acceptance data has been accumulated by this branch.

Results: Since July 1970 porcine xenograft cardiac valves have been implanted in 177 patients as either single or multiple replacements. One hundred-thrity-five patients have survived operation and have been followed for 3232 months. Early mortality is 12% and late mortality equals 13%. Anticoagulants are administered to patients with either a Starr-Edwards or Bjork-Shiley prosthesis in another position. Four of 80 (5%) patients with isolated xenografts have had emboli, 2 in the early postoperative period and 2 late. Eight of the remaining 97 (8%) mixed valve replacements have had emboli on anticoagulants. Death has not been attributed to thromboembolism in the isolated xenograft group. Hemodynamically the xenograft has been proven satisfactory in the mitral, tricuspid and aortic position. Fifteen patients have been followed for periods greater than 4 years and five of these patients for more than five years. Prosthetic dysfunction occurred in one patient 57 months following implant.

Porcine xenograft cardiac valves will continue to be implanted in the atrioventricular position routinely and in the aortic position in selected patients for further long term evaluation.

Publication:

McIntosh, C. L., Michaelis, L. L., Morrow, A. G., Itscoitz, S. B., Redwood, D. R., and Epstein, S. E.: Atrioventricular valve replacement with the Hancock porcine xenograft: A five year clinical experience. Surgery 78: 768-775, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02602-02 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cardiac Patient Data Profile

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI
Andrew G. Morrow, Chief, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

Data Management Branch, DCRT

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

SUMMARY OF WORK (200 words or less - underline keywords)

Approximately 4250 patients have undergone operation for acquired and congenital heart disease in this clinic. Pre- and postoperative hemodynamic, clinical assessment and long term follow-up has generated considerable data concerning survival, complications, and functional improvement following cardiac operation. Individuals who have undergone single or multiple valve replacement (in≃1100); mitral or aortic commissurotomy (in≃450) or coronary artery bypass grafting (in≃100) have been entered into a computer based program for the purpose of record keeping and analyses of survival, quality of life and complications following operation. The records are updated with each outpatient visit, hospital admission, or communication with either patient or physician. If a patient fails an appointment for a period of greater than 90 days, his name is automatically selected by the computer for more extensive follow-up. Life survival curves and histograms for the various cohorts of patients can now be directly plotted by the computer program. Causes of death, incidence of thromboembolism, anemia, hepatitis, or prosthetic dysfunction can also be retrieved in tabular form for any specific series of valves or particular operation. Multiple other studies have utilized this computer based program for hemodynamic incidence of complications, or long term follow-up data of various groups of patients who have undergone a cardiac operation.

1027

PHS-6040
(12-75)

Description: A computer based program has been established at NHLI which allows pre- and postoperative hemodynamic, clinical assessment, complications and long-term followup information concerning acquired and congenital cardiac patients to be stored, retrieved and analyzed. Retrospective hospital chart reviews will be done for patients already operated upon at NHLI and new patients will be entered into the data file at the time of admission to the Surgical Branch. Updating of individual files is done with each outpatient visit, hospitalization or communication concerning that patient. Specific group analyses will be performed every six months.

Results: Data compilation from hospital records has been completed in individuals who have undergone single or multiple valve replacement (in $\tilde{=}$1100). Life survival curves, causes of death, incidence of thromboembolism, anemia, hepatitis, prosthetic dysfunction or myocardial infarction (major complications) are being constructed and tabulated for the various cohorts of cardiac patients. All patients with acquired and congenital heart disease will be registered in this computer based program. Statistic analysis will be performed on the cohorts of patients with the specific prosthesis or type of operation being the variable. Survival, quality of life and incidence of complications will then be compared for the various types and combinations of values and operations.

The data is also stored in microfisch form for ready retrieval and ease of handling.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02603-02 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Surgical Management of Acquired Tricuspid Valve Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert H. Breyer, Surgeon, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
OTHER: Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1½	1	½

SUMMARY OF WORK (200 words or less - underline keywords)

The management of tricuspid valve disease associated with mitral or mitral-aortic disease continues to be a controversial subject. In 1967 the argument for conservative management was presented by this Clinic. Since that time, our approach to tricuspid valve disease has become more aggressive and an increasing number of patients undergo tricuspid valve replacement or annuloplasty. A retrospective review was undertaken to compare the results of conservative management vs. tricuspid annuloplasty vs. tricuspid valve replacement. During the years 1972-74, 76 patients were diagnosed to have tricuspid regurgitation and/or stenosis by palpation of the valve at the time of operation. Twenty-one patients did not undergo any tricuspid procedure, 30 patients underwent tricuspid annuloplasty, 25 underwent tricuspid valve replacement with porcine xenografts. A good clinical result was achieved in significantly more patients undergoing tricuspid valve replacement (80%) than in patients undergoing tricuspid annuloplasty (50%). Results indicate that an aggressive surgical approach is indicated in the management of tricuspid valve disease. Tricuspid valve replacement is shown to be superior to tricuspid annuloplasty.

1029

Description: During the years 1972-1974, 146 patients underwent mitral valve replacement, isolated or combined with aortic valve replacement. In 118 patients an intraoperative assessment of tricuspid valvular competence was made by digital palpation of the valve prior to the institution of cardiopulmonary bypass. In 76 of the 118 patients, tricuspid regurgitation of variable degree was evident.

In 21 patients, tricuspid regurgitation was judged to be mild, and no operative procedure on the tricuspid valve was carried out. Thirty patients were considered to have moderate tricuspid regurgitation and underwent suture annuloplasty of the tricuspid valve. The remaining 25 patients had severe tricuspid regurgitation and/or tricuspid stenosis; in these, the tricuspid valve was replaced with a Hancock porcine xenograft. Fourteen of the 76 patients (18%) died within 30 days of operation. Early mortality was highest among patients undergoing tricuspid annuloplasty (27%), 12% among patients undergoing tricuspid valve replacement, and 14% in patients in whom the valve was not treated operatively.

Mean length of followup has been 13 months. Forty-nine of the 76 patients underwent cardiac catheterization six months postoperatively; there were no significant differences among the three groups in mean right atrial or pulmonary arterial systolic pressure. However, a good clinical result (Functional Class I-II) was achieved in significantly more patients undergoing tricuspid valve replacement (80%) than in patients undergoing tricuspid annuloplasty (50%). Conservative management is justified in the patient with mild, presumably functional tricuspid regurgitation. In our experience, suture annuloplasty has not been effective in correction of tricuspid valvular regurgitation, and valve replacement with the Hancock porcine xenograft is now advocated when moderate or severe tricuspid regurgitation is evident when the valve is palpated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02605-08 SU

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

AEC-Arco Atomic Powered Pacemakers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Andrew G. Morrow, Chief, Clinic of Surgery, NHLI
Charles L. McIntosh, Senior Surgeon, Clinic of Surgery NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Surgery

SECTION

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INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

SUMMARY OF WORK (200 words or less - underline keywords)

Twenty-nine NU-5 series Arco atomic powered pacemakers continue to function without mechanical difficulties in dogs. Five humans have received Arco NU-5 series pacemakers; three continue to function well. The two Arco pacemakers removed from patients (secondary to skin erosion) demonstrate the undesirable design configuration of this pacemaker. One of these pacemakers also showed a predictable decline in pacing rate of 0.1 beat per week, with the rate finally stabilizing at 70. The concept of Plutonium^{238} as a power source for this cardiac device continues to be an excellent one. No radiation hazards have resulted from the use of this atomic powered cardiac pacemaker.

1031

PHS-6040
(12-75)

Description: Approximately 50 units of the NU-5 series were implanted in patients in ten cooperating units in the United States between March 1973 up to the current time. NIH initial quota of five NU-5 series pacemakers were implanted between April 1973 and December 1975. The Arco atomic powered pacemaker is a fixed rate device, and therefore we also elected to institute the insertion of Coratomic C-101 atomic powered pacemakers which is a demand unit. The atomic powered pacemakers continue to be implanted in patients who develop complete heart block following an intracardiac operation. Since the original protocol was submitted, the major changes in patient selection are extension of the upper age limit from 65 to 75 and allowing a threshold of 4 ma in a chronically implanted wire. The other major change is the followup period which now is as follows: 24 hours, one week, one month, 3 month, and 6 month intervals thereafter until removal of the pacemaker. Followup examination is both by clinic visit on a yearly basis, and ECG telephone transmissions as noted in the above time schedule. Thirty NU-5 series Arco atomic powered pacemakers were implanted in dogs between July and October of 1972 for long term laboratory evaluation.

Results: Five Arco NU-5 series pacemakers have been implanted since April of 1973. Three of these pacemakers remain implanted for cumulative experience of 81 patient months. One of the original implants was removed because of skin erosion and a predictable decrease in pacing rate of one-tenth of a beat per week. Arco predicted that this decrease would stop once the pacemaker reached 70, but we elected to remove the unit and replace it with a second NU-5 pacemaker which continues to function normally. The second Arco pacemaker was removed because of skin erosion which appears to be a common problem because of the design of the Arco pacemaker. The Arco pacemaker is also quite large compared to the Coratomic which is about the size of a pocket watch and has a much better design configuration. Two Coratomic pacemakers have been implanted to date since October 1975 and both units continue to function properly and have had excellent patient acceptance.

Twenty-nine NU-5 series Arco atomic powered pacemakers continue to function in dogs four years following implantation. The single failure occurred ten months following implantation. To date there have been no serious mechanical failures in clinical implants and no radiation hazards noted as a result of the use of atomic powered pacemakers.

Atomic powered pacemakers will continue to be employed when indicated in surgical patients with complete heart block, if they fit the selection criteria outlined in our original protocol and the revised protocol.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02617-02 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Extended Preservation of Dog Heart for Orthotopic Transplantation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald C. Watson, Surgeon, Clinic of Surgery, NHLI
T. L. Spray, Clinical Associate, Pathology, NHLI

COOPERATING UNITS (if any)
Section of Pathology

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-2/3	1	2/3

SUMMARY OF WORK (200 words or less - underline keywords)

In order to develop a better technique for myocardial preservation, the limits of high potassium arrest, using perfused and nonperfused models were tested. Dog hearts (N=35) were removed and preserved for 24 or 48 hours using an oxygenated hypertonic intracellular solution containing potassium phosphate, magnesium sulfate, glucose, and mannitol. Hearts were divided into groups and either nonperfused, intermittently perfused, or continuously perfused at 20 cm H_2O. Coronary flow, preservative pH and electrolyte concentrations were regularly monitored. After the period of preservation orthotopic transplantation was performed to evaluate cardiac function in a working state. After 24 hours of preservation all six continuously perfused hearts survived the initial 24 hour postoperative period and maintained excellent hemodynamic function. At 48 hours intermittent perfusion has provided better post-transplant function than continuously perfused hearts. The variables important to long-term preservation, optimum preservative ingredients and length of intermittent perfusion cycles are being investigated.

1033

Description: Preservation of a donor heart (DH) for greater than 12 hrs. has not usually resulted in consistent extended host survival after orthotopic cardiac transplantation (OCT). Heterotopic transplantation models have shown reliable myocardial viability but do not evaluate left ventricular volume load performance after preservation. Twelve dogs underwent OCT after DH preservation for 24 hrs. in a hypothermic, hypertonic intracellular solution containing potassium phosphate, magnesium sulfate, glucose and mannitol. All DH were removed in an empty beating state, gradually cooled to 3°C. and irrigated with one liter of perfusate at 20 cm H_2O pressure. DH were divided into two equal groups: continuous oxygenated perfusion and no further perfusion. After 24 hrs. OCT was performed. Survivors were killed at 24 hrs. All 6 perfused and 4 nonperfused DH survived a 24 hr. postoperative period. Survivors had normal cardiac output, right and left atrial pressures and aortic pressures. After perfusion, perfused hearts showed a 34% weight gain ($p < .002$) and an 18% increase in coronary resistance. After transplantation, all perfused hearts demonstrated endocardial petechiae ($p < .002$), normal left ventricular wall thickness and a return toward preperfusion weight. All nonperfused hearts had severe damage with diffuse transmural hemorrhage ($p < .002$) and variable left ventricular wall thickness and weight. Light microscopic examination of the nonperfused donors showed multiple areas of focal necrosis with loss of intracellular organization and endocardial cellular destruction. Perfused hearts had less evidence of myocyte damage. Oxygenated perfusion with a hypothermic, hypertonic intracellular solution allowed 100% graft survival after orthotopic transplantation and no obvious transmural damage after 24 hrs. of preservation.

To explore the limits of one technique, 23 dogs underwent OCT after DH preservation for 48 hrs in this preservative and steroids. All DH were removed after pretreatment with steroids and irrigation with hypothermic normal saline containing potassium chloride (30 mEq/L). DH were gradually cooled to 3°C and irrigated with one liter of oxygenated intracellular solution at 20 cm H_2O pressure. One group of hearts (N=17) underwent continuous oxygenated perfusion. A second group (N=6) had 15 min cyclic intermittent oxygenated perfusion. After 48 hrs OCT was performed. Survivors were killed at 24 hrs. All 6 (100%) intermittently perfused and 4 (24%) continuously perfused DH survived a 24 hr postoperative period ($p < .005$). Survivors had a normal cardiac output, aortic pressure and right atrial pressure. Left atrial pressures were normal in half of each group of survivors. After perfusion, continuously perfused hearts showed an 86% weight gain and a 105% increase in coronary artery resistance. Intermittently perfused hearts gained 58% and 72% respectively ($p < .01$). After transplantation, the left ventricle of all survivors with normal left atrial pressures showed mild subendocardial hemorrhage and a return toward preperfusion weight. Other survivors had mild transmural hemorrhage, sloughing of a thin subendocardial layer and a smaller return toward preperfusion weight. Nonsurvivors had severe diffuse transmural hemorrhage and no weight change. Intermittent oxygenated perfusion with a hypertonic, hypothermic intracellular solution allowed 100% graft survival and mild transmural damage after 48 hrs of preservation and OCT. Continuous oxygenated perfusion did not provide as much protection.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02620-02 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effects of Isoproterenol and Dopamine in Regional Myocardial Blood Flow

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
Robert A. Guyton, Surgeon, Clinic of Surgery, NHLI
Robert H. Breyer, Surgeon, Clinic of Surgery, NHLI
Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI

OTHER: Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of isoproterenol and dopamine on regional myocardial blood flow was studied in ten open-chest dogs after proximal circumflex coronary artery stenosis. Regional myocardial flows were determined with radioactive microspheres.

Both drugs increased mean aortic flow and myocardial contractility. Isoproterenol caused relative subendocardial ischemia in the myocardium with compromised coronary flow. Dopamine did not produce regional myocardial ischemia. It is concluded that dopamine is the preferable inotropic drug in treating low cardiac output in patients with coronary artery disease.

1035

Description: The effects of isoproterenol and dopamine on regional myocardial blood flow were studied in 10 open-chest dogs after acute proximal circumflex coronary artery stenosis. Blood flow was determined using the radioactive microsphere technique. Isoproterenol led to a homogeneous increase in blood flow in the normal myocardium. In the myocardium with compromised coronary blood flow, isoproterenol led to relative subendocardial ischemia. This occurred despite increased aortic flow and peak left ventricular dp/dt. Dopamine also increased aortic flow and peak left ventricular dp/dt, but did not cause regional myocardial ischemia. The findings suggest that dopamine is the preferable inotropic agent in treating low cardiac output in patients with significant coronary artery disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02621-02 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Distribution of Blood Flow Before and After Repair of Coarctation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI

OTHER: Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1¼	3/4	½

SUMMARY OF WORK (200 words or less - underline keywords)

Two postoperative complications which may occur after repair of coarctation of the aorta are hypertensive crisis and mesenteric vasculitis. The etiology and mechanism of these potential hazardous complications, which may be related to sympathetic rebound phenomena, is difficult to establish and investigate because of the difficulties in developing an animal model and determining regional blood flow.

A model of coarctation of the aorta has been developed by constricting the descending thoracic aorta with tape tied over felt pads. The animal models were allowed to mature, each retaining a significant gradient across the coarctation. In these animals regional blood flow distribution will be determined (by radioactive microspheres) to myocardium, kidney, GI tract, and skeletal muscle above and below the coarctation before and after surgical repair of the coarctation in the awake state. Also, coincident with these blood flow measurements, serum epinephrine and norepinephrine levels will be obtained for determination and used as an index of sympathetic tone. It is hoped that this study will provide some insight into the etiology and mechanism of the postoperative complications that occur with surgical correction of coarctation of the aorta.

1037

PHS-6040
(12-75)

Description: Postoperative complications which may occur after surgical repair of coarctation are severe and may be related to sympathetic rebound pneumonia. This study attempts to explore a possible etiology and mechanism of these severe postoperative problems.

Adult American foxhounds (18-25 Kg) were used in this study. Each animal underwent a left thoracotomy through which a coarctation was made by tying umbilical tape over felt pads passed around the aorta. The coarctation was just distal to the left subclavian artery. Pressure measurements proximal and distal to the coarctation were obtained intraoperatively as an aid in constructing the coarctation. Each animal underwent catheterization at 6 weeks and 3 months. The mean gradient across the coarctation was greater than 30 mm. Hg on each occasion.

Presently there exists two groups of coarctation models; one approximately 18 months since preparation, and the other approximately 12 months. Regional blood flow to several organs will be measured before and after surgical repair of the coarctation in the awake state with the use of radioactive microspheres. Thus, each animal will undergo additional preparation approximately ten days before the surgical correction of the coarctation. This will involve implantation of a right atrial line and an arterial line proximal and distal to the coarctation.

Radioactive microspheres will be used to study regional organ blood flow pre- and postcoarctation repair in the awake animal. Blood flow will be determined two hours before correction, and 6, 12, and 24 hours postcorrection of the coarctation. Thus, with tissue specimen from the heart, kidney, gut, and skeletal muscle proximal and distal to the coarctation, regional organ blood flow can be determined at these times. Additionally and coincident with these times, serum samples will be obtained for determination of serum epinephrine and norepinephrine levels. These results will be used as an index of sympathetic tone.

Results: A colony of coarctation of the aorta exists in two groups. Each group has matured: Group I to 18 months and Group II has matured to 12 months. Each animal has undergone catheterization at 6 weeks and 3 months and each animal had a surgically significant coarctation of the aorta.

Each animal is presently undergoing preparation prior to the repair of the coarctation. No animal has undergone the repair. This study was assigned to investigate a possible etiology and mechanism of the postoperative patient and hypertensive crisis and mesenteric vasculitis. Although not completed, it is hoped that some insight will be provided by this study into the pathophysiological factors which occur after surgical repair and the coarctation of the aorta.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02623-16 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Operative Treatment of Patients with Obstructive Cardiomyopathies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Andrew G. Morrow, Chief, Clinic of Surgery, NHLI

OTHER: Donald C. Watson, Surgeon, Clinic of Surgery, NHLI
Stephen E. Epstein, Chief, Cardiology Branch, NHLI
Walter L. Henry, Senior Investigator, Cardiology Branch, NHLI

COOPERATING UNITS (if any)
Cardiology Branch

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1½	1	½

SUMMARY OF WORK (200 words or less - underline keywords)

A major interest of the Surgery Branch since 1960 has been the development of operative methods for the relief of obstruction in patients with idiopathic hypertrophic subaortic stenosis (IHSS). As of March 31, 1976 132 patients have been operated upon and 106 have been evaluated in detail postoperatively. At operation a wedge of left ventricular septum is resected. After operation there was a distinct symptomatic improvement in all survivors. Cardiac catheterization has confirmed relief of obstruction in these patients. Nine patients died at the time of operation. Long-term followup has not demonstrated recurrence of obstruction. This continuing experience demonstrates that operation with left ventricular septal wedge resection in symptomatic IHSS patients with documented left ventricular outflow obstruction is low risk and provides symptomatic and hemodynamic improvement.

1039

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02624-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Radioisotopic Quantitation of Left to Right Atrial Cardiac Shunts in Dogs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald C. Watson, Surgeon, Clinic of Surgery, NHLI
P. O. Alderson, Major, USAF, MC, Naval Medical Center

COOPERATING UNITS (if any)
Armed Forces Radiobiology Research Institute, Naval Medical Center

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

SUMMARY OF WORK (200 words or less - underline keywords)

Quantification of intracardiac shunts in congenital heart disease in man has usually been achieved in the past by the invasive techniques at catheterization. Recently radioisotopic techniques have been useful in detecting and quantitating these shunts noninvasively. Little effort has been directed toward studying the effect of simultaneous congenital defects on reproducibility and accuracy of shunt ratio quantified with radioisotopic technique. A group of dogs (N=16) is being examined after surgical creation of an atrial septal defect and again after creation of a pulmonary artery band or mitral valve incompetence. Each shunt ratio determination is being performed with radioisotopic, electromagnetic flow probe and oximetric techniques to determine reproducibility and accuracy of each method. Determination of the effect of a second intracardiac defect on the reproducibility and accuracy of each technique is in progress. This noninvasive technique may allow more reliable shunt ratio determination.

1041

Description: Since 1960, 132 patients have been operated upon for idiopathic hypertrophic subaortic stenosis. Most patients with this disease are asymptomatic or benefit significantly from nonoperative therapy. However, a subgroup (about 15%) of patients remain severely symptomatic after maximal medical therapy and required operation for relief of resting or provocable left ventricular outflow tract obstruction.

All 132 operative patients were severely incapacitated: New York Heart Association functional class III or IV. One hundred three patients had severe left ventricular outflow obstruction at rest, and 29 had provocable obstruction. At operation two longitudinal left ventricular septal myotomies are made and the interposed muscle removed. Nine patients have died at operation (7%). Nine patients have died late after operation, six of causes unrelated to their heart disease.

All surviving patients have described an improved functional status. Of 106 patients followed postoperatively, 104 were functional class I or II. Of 87 patients with preoperative osbtruction at rest, 72 had no resting postoperative obstruction, 10 had pressure gradients less than 25 mm Hg and the remaining 5 had gradients greater than 25 mm Hg but were significantly decreased from preoperative evaluation. Obstruction after operation tended to decrease with time. No recurrence of obstruction has been documented by catheterization or echocardiogram. Nineteen patients with provocable obstruction showed decreased response to provocative maneuvers after operation. Study is currently in progress regarding this operation in elderly patients and the effects of operation on long-term arrhythmias.

These results demonstrate that operation with left ventricular septal wedge resection in symptomatic IHSS patients with left ventricular outflow obstruction, resting or provocable, is low risk and provides uniform symptomatic and hemodynamic improvement.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02625-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Effect of the Angle of Aortic Anastomosis on Histologic Changes in Venous Coronary Artery Bypass Grafts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert H. Breyer, Surgeon, Clinic of Surgery, NHLI
OTHER: Thomas L. Spray, Staff Associate, Pathology, NHLI
David G. Kastl, Surgeon, Clinic of Surgery, NHLI
Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
Section of Pathology

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	2/3	1/3

SUMMARY OF WORK (200 words or less - underline keywords)

Early and late histologic changes in saphenous vein coronary artery bypass grafts have been reported by numerous authors. Subendothelial cellular proliferation has led to graft failure in some cases.

A study was undertaken to determine whether the angle of the aorto-saphenous vein anastomosis influences the degree of subendothelial proliferation. Thirteen dogs survived aortocoronary bypass to the left circumflex coronary artery. The angle of aortic anastomosis was acute in 5 dogs, perpendicular in 2 dogs, and obtuse in 6 dogs. Angiograms demonstrated open grafts at least six months following operation in 12 of the 13 dogs. All the dogs have been killed, and the hearts perfusion-fixed. Histologic examination of the vein grafts is pending.

1042

Description: Early and late histologic changes in saphenous vein coronary artery bypass grafts have been reported by numerous authors and in some cases subendothelial cellular proliferation has led to graft failure. These changes have been ascribed to a number of technical factors (including vein length and diameter, geometry of proximal and distal anastomosis, handling of the vein during harvesting), as well as to the effect of ischemia, arterial pressure, and available distal coronary bed. Indeed, experimental models have demonstrated the importance of distal runoff, arterial pressure, and the size of the vein graft. It has not been documented that the angle of the aortic vein anastomoses can influence late vein graft changes, although a theoretical basis for such an effect has been proposed.

A study was undertaken to determine whether the angle of aorto-saphenous vein anastomosis influences the degree of subendothelial proliferation. Thirteen of 16 dogs survived aortocoronary bypass to the left circumflex coronary artery. The proximal anastomoses were constructed by one of three different methods. In 2 dogs, the vein graft was cut at an 90^{o} angle and sutured to circular ostia in the aortic wall, creating an aorta-graft angle of 90^{o}. In 13 dogs, the vein graft was cut at a 45^{o} angle; and then sutured to a longitudinal slit in the aortic wall. In 5 of these dogs, the vein graft was directed distally toward the circumflex artery, so as to create an angle of $45\text{-}60^{o}$ with the ascending aorta. In the remaining 6 dogs, the vein graft was directed cephaled and then looped gently back toward the circumflex artery, creating an aortic-graft angle of 120^{o} - 135^{o}.

Results: Cineangiogram demonstrated open grafts at least 6 months (6 - 21 months) following operation in 12 of the 13 dogs. All the dogs have been killed, and the hearts perfusion-fixed. Histologic examination of the vein grafts is pending.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02626-01 SU

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Selective Retrograde Revascularization of the Coronary Vein: An Ischemic Insult?

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glen R. Rhodes, Surgeon, Clinic of Surgery, NHLI

OTHER: Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH

Surgery

SECTION

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INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1½	1	½

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of selective retrograde revascularization of the coronary vein (SRRCV) and selective coronary vein occlusion (SVC) on both normal and ischemic myocardium were evaluated in a canine model (10 animals). The distribution of myocardial blood flow (MBF) and multiple hemodynamic parameters were measured before and after occlusion of the main left anterior descending coronary artery (LADCA) and after 5 minutes and 30 minutes of SSRCV to the great cardiac vein. SCVO decreases mean MBF in H and L myocardium with increased distribution to the endocardium. This decrease is significantly greater in L (distal) compared to (H) proximal myocardium. SRRCV to normally perfused myocardium in H did not significantly change MBF; however, MBF remained less in H than in L myocardium. SRRCV to acutely ischemic myocardium L restored less than 50% of the decrease in MBF. SRRCV does not appear to greatly enchance MBF to ischemic areas and may significantly reduce MBF on a basis of venous occlusion alone.

1044

Description: Selective retrograde revascularization of the coronary vein (SRRCV) has been advocated for patients with diffuse coronary disease; however, the benefits of this procedure remain unproven. The effects of selective coronary vein occlusion (SVCO) and SRRCV on both normal and ischemic myocardium were evaluated in a canine model (10 animals). Distribution of myocardial blood flow (MBF) as determined with radioactive microspheres, mean arterial (AP) and left atrial pressure (LA), and heart rate (HR) were serially measured. SRRCV was performed with a shunt interposed between the ascending aorta and great cardiac vein (GVC), which drains the left anterior descending coronary artery (LADCA) distribution. The vein was ligated distal to the shunt. With the shunt closed, measurements were made before and after ligation of the mid LADCA. With the mid LADCA occluded, measurements were made after 5' and 30' of SRRCV. The myocardium was divided into control circumflex (C), high LADCA (H), above the arterial occlusion with arterial and SRRCV flow, and low LADCA (L), below the arterial occlusion with SRRCV only.

SCVO decreased mean MBF 22% ($p > .05$) in H, and 42% ($p < .025$) in L compared to C. MBF in H and C areas, AP, LA, and HR did not significantly change during the study. Occlusion of the mid LADCA decreased mean MBF 78% in the L area. SRRCV restored only 47% of this decrease after 5' and only 37% after 30'. There was proportionately greater endocardial than epicardial MBF in H and L ($p < .05$) compared to C throughout the study.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02627-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
An Experimental Evaluation of Coronary Air Embolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glen R. Rhodes, Surgeon, Clinic of Surgery, NHLI

OTHER: Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	¼

SUMMARY OF WORK (200 words or less - underline keywords)

Coronary air embolism (CAE), a known but infrequent complication of cardiac bypass, was studied as an ischemic injury in two groups of sheep. The distribution of myocardial blood flow (MBF - measured with radioactive microspheres) and hemodynamic parameters were measured before and after injection of 1 ml of air into the proximal circumflex coronary artery. MBF was compared in embolizaed and normal myocardium. CAE caused marked transient decreases in mean MBF with redistribution (R) of blood flow away from the subendocardium. A hypermic recovery period was seen within 15 minutes of CAE with recovery of all measured parameters except dp/dt within 30 minutes. Rapid restoration of arterial pressure with dopamine resulted in significantly increased mean MBF, initially associated with (R). (R) is superseded within 5 minutes by subendocardial hyperemia with continued dopamine infusion.

1046

PHS-6040
(12-75)

Description: Coronary air embolism (CAE) may occur as a complication of cardiac bypass. This form of ischemic injury was studied in two groups of sheep in which 1 ml of air was injected into the proximal circumflex coronary artery. The distribution of myocardial blood flow (MBF), as determined with radioactive microspheres, mean arterial pressure (MAP), mean left atrial pressure (LA), and the first derivative of left ventricular pressure (dp/dt), were measured serially in each study. The myocardium supplied by the embolized circumflex artery was divided into proximal (P) and distal (D) sections, which were compared to left anterior descending (LAD) myocardium.

Measurements were made in Group A (7 animals) before and 3', 15', and 30' following CAE. In Group B (6 animals) CAE was treated with dopamine infusion (10-25 mEq/Kg/min) begun 3' after embolism, with measurements made before, 2', 5', and 10' following CAE. In Group A mean MBF decreased significantly in embolized myocardium, 45% in P and 22% in D with redistribution (R) of blood flow away from the subendocardium, i.e. reduced subendocardial/epicardial blood flow ratio. Mean MBF increased 51% in LAD myocardium despite a decrease in MAP from 84 to 59 mm Hg, an increase in LA from 6.8 to 16.5 mm Hg, and a decrease in dp/dt from 1350 to 620 mm Hg/sec. Marked transmural and especially subendocardial hyperemia were present 15' after CAE in the embolized area of myocardium. In Group B, dopamine rapidly restored MAP to control by 5' and 26% above control values by 10' following CAE. This intervention almost tripled mean MBF, accompanied by (R) at 5', and by subendocardial hyperemia at 10'.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02628-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Comparison of Pericardial Tubes, Composite Spark's Mandril Grafts, and Azothioprine Treated Vein Homograft for Aorto-coronary Grafting

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glen R. Rhodes, Surgeon, Clinic of Surgery, NHLI

OTHER: Donald C. Watson, Surgeon, Clinic of Surgery, NHLI
David G. Kastl, Surgeon, Clinic of Surgery, NHLI
David L. Gregg, Surgeon, Clinic of Surgery, NHLI
Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI
G. Melville Williams, Senior Surgeon, Surgery Branch, NCI

COOPERATING UNITS (if any)
Surgery Branch, NCI

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1½	1	½

SUMMARY OF WORK (200 words or less - underline keywords)

This report summarizes several investigations into tissue substitutes for use as aorto-coronary grafts when saphenous veins or internal mammary arteries are unavailable. Three types of tissue conduits have been studied in chronic experiments up to a period fo eight months. Adult foxhounds underwent standard sterile cardiac bypass with placement of grafts between the aorta and circumflex coronary artery. Group A consisted of 14 animals who received autologous pericardial tubes. Group B consisted of 6 dogs receiving Spark's mandril grafts. Group C consisted of 20 dogs in which homologous saphenous veins were used in conjunction with low doses of azothioprine. Group A grafts invariably occluded by 3 1/2 months in two patterns: early diffuse thrombosis of the entire graft or late thrombosis of an established graft at distinct sites of graft degeneration, ulceration, and annular narrowing. All Group B grafts clotted within one hour following cardiac bypass. In Group C after adjustment of azothioprine doses, these grafts appear to have an early (3 month) patency rate of 80%. These animals will be followed up to a year to evaluate patency. Histologic techniques including Barr body analysis will be used to serially evaluate the degree of host replacement of the donor grafts.

1048

PHS-6040
(12-75)

Description: The increasing number of patients undergoing aortocoronary revascularization for obstructive coronary disease has sharpened interest in the development of alternative small vessel conduits as aortocoronary grafts. With the knowledge that small caliber prosthetic grafts uniformly thrombose in past experiments, our investigations were restricted to tissue grafts of three types: Group A consisted of 14 animals receiving autologous pericardial tubes, which were hand sewn tubes averaging 10-12 cm. in length tapering from 3 mm. distally to 5 mm. proximally. Multiple preliminary experiments were required to develop adequate means of protecting the pericardium during fabrications of the tubes. Group B consisted of dogs receiving composite Spark's mandril grafts which were impregnated with fibrin prior to eight weeks of subcutaneous implantation. Two grafts were 3mm., two were 4 mm., and two tapered from 4-6 mm. in diameter. Group C consisted of 20 dogs in which homologous saphenous veins were employed. These animals were treated with low dose azothioprine, 1.5 mg./kg. Angiograms were perfomred serially in all animals to confirm patency. Routine histologic techniques were used to evaluate grafts in all groups and Barr body analysis will be used to serially evaluate the degree of host replacement of donor grafts in Group C.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02629-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Evaluation of Hemolysis Following Atrioventricular Replacement With Porcine Xenograft (Hancock) Valves

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glen R. Rhodes, M. D., Surgeon, Clinic of Surgery, NHLI

OTHER: Charles L. McIntosh, M. D., Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
½	½	

SUMMARY OF WORK (200 words or less - underline keywords)

Hematologic and hemolysis studies were performed in 22 patients following xenograft atrioventricular valve replacements to evaluate the degree of hemolysis associated with these valves. Patients were studied 6-62 months postoperatively (mean 30.5 months). Preoperative mean hematologic values were: Hematocrit 39.4, reticulocyte index 1.3%, platelet count 281 x $10^3/mm^3$ and LDH 234. The LDH was greater than 400 in five patients. The postoperative mean values were: Hematocrit 40.8, reticulocyte index 1.2%, platelet count 227 x $10^3/mm^3$ and LDH 204. Hemolysis studies including serum hemoglobin, and serum haptoglobin failed to indicate hemolysis in any patient. Postoperative iron studies were normal for the majority of patients. Only two patients had mild anemias, and their hemolysis studies were normal. Five patients had significantly elevated LDH values preoperatively which returned to normal following operation. Our evidence suggests that the glutaraldehyde fixed porcine xenograft provides excellent hemodynamic function without significant thromboembolism or chemically detectable hemolysis. Presently, it is our valve of choice for all atrioventricular replacements.

1050

Description: Intravascular hemolysis is a frequent and occasionally serious complication of prosthetic valvular replacements. A reduction in thromboembolism and hemolysis has been sought through clinical trials of new and modified prosthetic and tissue valves. Perhaps the most widely used tissue valve has been the glutaraldehyde-fixed porcine xenograft (Hancock) on a Dacron-covered flexible stent. In five years of experience, this valve has provided excellent hemodynamic function without significant thromboembolism. Also, it has been our clinical impression that negligible hemolysis occurs with this valve. To test this hypothesis, hematologic studies were performed on 22 patients following atrioventricular valve replacements.

Twenty-two patients who had undergone porcine xenograft replacement of one or more atrioventricular valves were investigated, either during their admission for postoperative catheterization or during their annual clinic visit. Blood was drawn for measurement of a complete blood count, reticulocyte index, red cell indices, and platelet count. LDH and total billirubin were determined by standard methods. Serum hemoglobin, serum haptoglobin, serum iron, total iron binding capacity, and iron saturation were also measured.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02630-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Hemodynamic and Clinical Results of Triple Valve Replacement: Comparison of All-Mechanical with Porcine Xenograft Replacement

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glen R. Rhodes, Surgeon, Clinic of Surgery, NHLI

OTHER: Charles L. McIntosh, Senior Surgeon, Clinic of Surgery, NHLI
Andrew G. Morrow, Chief, Clinic of Surgery, NHLI
Samuel B. Itscoitz, Ass't Head, Cath Lab, Cardiology Branch, NHLI

COOPERATING UNITS (if any)
Cardiology Branch

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
½	½	

SUMMARY OF WORK (200 words or less - underline keywords)

This study summarizes the preoperative and postoperative clinical and hemodynamic results in 26 patients undergoing simultaneous triple valve (aortic, mitral, tricuspid) replacement (TVR) between 1966-1975. Ten patients who received only mechanical prosthetic replacements (Group I) are compared with fifteen who received porcine xenograft (Hancock) replacements of all atrioventricular valves (Group II). The patient profiles of both groups including sex, age, severity of symptoms, cardiac lesions, and preoperative hemodynamic parameters were similar. Severe cardiac decompensation was evidenced by pulmonary arterial and venous hypertension with low cardiac indices in both groups. Total operative and bypass time and duration of inotropic and respiratory support were shorter, and hemodynamic improvement was better in Group II compared with Group I following TVR. The requirements for temporary pacing and incidence of heart block were greater in Group II. Early mortality was Group I, 33%, Group II, 18.8%. The late survival rates for operative survivors were Group I, 58% and Group II, 93%. There have been multiple late complications including thromboembolisn in Group I and none in Group II. Four of 7 Group I and 12/13 Group II operative survivors have improved at least 2 functional classes, enabling them to return to employment or resume household duties.

105x

Description: This study reviews all patients undergoing simultaneous replacement of aortic, mitral, and tricuspid valves between 1966-1975. Group I consisted of 10 patients undergoing operation between 1966-1972 in which each patient received three mechanical prostheses. Group II consisted of 16 patients who underwent operation between 1970-1975. Each patient received porcine xenograft (Hancock) replacement of both atrioventricular valves as well as a xenograft aortic replacement in two patients. Mechanical prosthetic valves were employed for 14 of 16 aortic replacements in Group II. Preoperative evaluation included preoperative catheterization and assessment of mean age, sex, average duration of symptoms, functional class, and hemodynamic lesions. Intraoperative evaluation included measurement of operative time, bypass time, and aortic cross-clamp time. The postoperative electrocardiographic study and duration of inotropic and respiratory support were assessed. Complete followup in all patients included postoperative catheterization in 4/10 Group I and 13/16 Group II patients.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 02631-01 SU

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

pCO_2 as Determinant of Coronary Blood Flow

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI

OTHER: Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI
Donald E. Syracuse, Surgeon, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI

Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1¼	3/4	¼

SUMMARY OF WORK (200 words or less - underline keywords)

The concentration of CO_2 has clearly been shown to have a direct effect on some vascular beds, for example, on the cerebral vasculature. Its effects in others, as in the coronary bed, is still not completely defined. In order directly to quantitate the effect of pCO_2 while maintaining constant pH and pO_2 on regional myocardial blood flow, the radioactive microsphere technique was used.

1054

PHS-6040
(12-75)

Description:

I - Uncontrolled State - this group consisted of 8 adult foxhounds who underwent continuous measurements of BP, HR, cardiac output, left circumflex coronary artery flow, LV dp/dt and LAP while the experimental interventions were performed. The concentration of CO_2 was increased by infusing into the Harvard respirator a gas mixture of 15-30% CO_2 - 85-70% O_2, while the acidosis was corrected with appropriate doses of $NAHCO_3$. The pCO_2 was likewise decreased by hyperventilating the animal until the desired state was achieved. The corresponding alkalosis was easily corrected with a solution of 0.5N HCl. Frequent blood gas measurements were conducted to determine the appropriate time for study.

The four injections of the radioactive microspheres were made at a control basal state, after one intervention (either producing hyper - or hypocapnea), after a second control or basal state had been achieved, and after the second intervention.

II - Controlled State - this group will consist of a similar number of dogs who will undergo same protocol, however, they will be placed on right heart bypass to control preload. A C-clamp will be placed on the descending aorta to control afterload and they will be paced.

Results:

I - Uncontrolled State - preliminary results show a statistically significant increase in flow in both endocardial (1.087 cc/min/gm vs. 2.64 cc/mm/gm) ($p < .005$) and epicardial layers (1.10 cc/mm/gm vs. 2.34 cc/mm/gm) ($p < .0010$) with no significant change in the endocardial/epicardial ratio (.976 vs .99) when the concentration of CO_2 was increased to levels between 55-90 mm Hg.

When the concentration was decreased to levels around 15 mm Hg there was no significant change to both layers: endo (1.06 vs 1.05) and epi (1.09 vs 1.12). Comparison of the two control periods shows no significant change between them (endo [1.08 vs 1.06], epi [1.10 vs 1.09]) assuring us of similarities between the two basal states.

Hemodynamic comparison between control and the hypercapneic state showed no significant changes in BP, HR, DP/DT and LAP and statistically significant changes in cardiac output ($p < .001$) and circumflex flow ($p < .001$). The hypocapniec state produced significant changes in BP ($p < .001$), HR ($p < .001$) and circumflex flow ($p < .005$) whereas BP and circumflex flow was reduced and heart rate increased. Cardiac output, DP/DT and LAP were not changed. No data are yet available for II-Controlled State.

In conclusion, it is hoped to show that increased pCO_2 increases myocardial blood flow significantly, and that it may be used as a therapeutic modality.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02632-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Intraoperative, Intracardiac Echo Measurement of the Septum in IHSS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLI
David G. Kastl, Surgeon, Clinic of Surgery, NHLI
Walter Henry, Senior Investigator, Cardiology Branch, NHLI
Andrew G. Morrow, Chief, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
Cardiology Branch, IR, NHLI

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/6	1/6	

SUMMARY OF WORK (200 words or less - underline keywords)

Left ventricular myotomy and myectomy has been shown as an effective operation for selected patients with IHSS. With increasing patient referrals, more centers are performing the operation, and it may prove helpful to obtain exact measurements of septal thickness during the myectomy. Theoretically, a servosystem could be designed, which would allow an exact depth to the myectomy, yet reliably avoid septal perforation and the creation of a ventricular septal defect. In this study septal thickness is measured by a specially designed echo probe which is inserted into the left ventricular outflow tract at the time of operation (myotomy and myectomy) for IHSS.

1058

Description: A 2.5 mhz echo transducer on a flexible probe has been designed for measuring intraoperative, intracardiac septal dimensions. When the sterilized probe is inserted into the left ventricular outflow tract, echos are obtained from the septum, which allows an exact determination of septal thickness. Permanent record of the echo measurement is obtained with a Honeywell 1856 Visicorder.

Results: Preliminary results (10 patients) indicate a close correlation between preoperative and intraoperative septal dimensions. The trough and remaining septum are clearly defined and easily measured. The operation as currently performed, creates a remarkably uniform-sized trough, not adjusted for varying septal thickness. We plan to follow each patient through a six month catheterization with interest in correlating late catheterization data with intraoperative echo measurements of the extent of myectomy.

Septal thickness at the time of operation for IHSS may be measured with an echo probe. It is hoped that knowledge of the remaining septal thickness may further improve the results of the operation and eliminate iatrogenic VSD as a complication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02633-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Improved Subendocardial Perfusion with Ligation of the Coronary Sinus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI
Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLI
Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2/3	1/3	1/3

SUMMARY OF WORK (200 words or less - underline keywords)

In 1935, Claude S. Beck recommended ligation of the coronary sinus as part of an operation to improve myocardial blood supply. In the present studies this procedure was evaluated by modern methods for measurement of total and regional myocardial perfusion and left ventricular performance. Coronary sinus ligation acutely decreases total LV perfusion, but, due to flow redistribution, the subendocardium is spared. In fact, after 30 min. the favorable flow redistribution to the subendocardium continues, at a time when LV perfusion returns to normal. It may be possible to improve blood flow to the subendocardium by elevating pressure in the coronary sinus.

1058

Description: Ten dogs underwent coronary sinus ligation (C.S.L.), and in 5 ligation of the anterior cardiac veins (L.A.C.V.) was also carried out subsequently. Radioactive microspheres were injected under four experimental conditions for each dog and used to determine regional myocardial perfusion. Hemodynamic measurements (H.R., BP, LAP, LV dp/dt max.) were made with each injection.

Results: C.S.L. increased the sinus pressure from a mean of 4.4 to 36.5 mm. Hg. Total LV perfusion decreased acutely following ligation (106.4 to 87.4 cc/100 Gm/min) but returned to normal within 30 min. Despite the acute drop in LV perfusion, the subendocardium was spared a decrease in its perfusion (37.9 vs. 35.7 cc/100 Gm/min). In fact, there was a significant ($p < .05$) enhancement of subendocardial perfusion at 30 min (37.9 vs. 44.2 cc/100 Gm/min). The improved subendocardial flow continued at a time after total LV perfusion had returned to normal. Hemodynamic functions were unchanged. L.A.C.V. combined with C.S.L. further decreased LV perfusion, abolished the improved subendocardial perfusion, and depressed left ventricular function significantly.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02634-01 SU

PERIOD COVERED July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

A New Design for an Emergency Transthoracic, Intramyocardial Pacing Wire

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLI
Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI
Donald C. Watson, Surgeon, Clinic of Surgery, NHLI

OTHER: George Thomas, Head, Bldg 10 Fabrication Group, Instrument Sec., BEID
John Clark, Instrument Fabrication Section, BEID
John Boretos, Instrument Fabrication Section, BEID

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
¼	¼	

SUMMARY OF WORK (200 words or less - underline keywords)

Emergency transthoracic pacing wires should be easy to insert, pace reliably, and pose miminal risk to the patient. The wires currently used for this purpose are inserted into the ventricular cavity and pace the endocardium. The major risks with these systems are electrode tip migration within the ventricular cavity, hemorrhage from cardiac puncture, and thrombus formation on the wire. This study presents a prototype for an alternate approach to emergency transthoracic pacing which may reduce these risks.

1060

PHS-6040
(12-75)

Description: The basic design is a four pronged, braided Elgiloy, spiral wire which paces the myocardium and does not enter the ventricular cavity. The prongs retain a "memory" for the fully expanded position, but are flexible and the entire apparatus may be loaded into an 18-gauge cannula. When advanced into the heart through this cannula, the wire seeks to return to its open, spiral shape and embeds in the myocardium.

Results: Fifteen foxhounds were studied under varied pacing schedules. All dogs survived the wire implantations and removals. The wire proved stable over a three day test period and threshold changes reached a plateau of 2.7 ma (mean) within 48 hours. During insertion in open chest dogs, transient ventricular premature beats occurred in 28%, subepicardial hemorrhage greater than 5 mm occurred in 6%, and a nonlethal myocardial perforation occurred in 2%. When dogs came to necropsy, the wire was found well embedded in myocardium with minimal evidence of injury. The electrodes coursed in a plane 3 mm below the epicardium, thus sparing 5-8 mm of myocardium from electrode intrusion.

The four pronged, spiral wire has two distinct advantages. First, it paces reliably because its electrodes are embedded in muscle. Second, it penetrates, rather than perforates, the myocardium. The endocardium is not violated and, therefore, the risk of hemorrhage, infection, and thrombosis should be lessened. Clinical trials are now planned for the new pacing wire which has proven reliable and safe in dogs.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02635-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Effects of Topical Hypothermia vs. Coronary Artery Perfusion on Regional Myocardial Blood Flow

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI

OTHER: Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI
Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1½	1	½

SUMMARY OF WORK (200 words or less - underline keywords)

The best method for protecting hypertrophied hearts during cardiopulmonary bypass at the present time is controversial among cardiac surgeons. Myocardial blood flow was studied in two groups of dogs with hypertrophied hearts using the radioactive microsphere technique before and after cardiopulmonary bypass.

1062

PHS-6040
(12-75)

Description: All the dogs used in this study were volume-induced hypertrophy models, created either by bilateral femoral artery to femoral vein shunts or by aortocaval shunts. Group I consisted of nine of these dogs who underwent cardiopulmonary bypass for 100 minutes with moderate systemic hypothermia (30°C), induced ventricular fibrillation, and coronary artery perfusion. Heart rate, $\overline{BP}$, Na^+, K^+, and arterial blood gases were recorded pre-bypass and post-bypass. The flow of the pump was set to maintain a perfusion pressure of around 75-80 mm. Hg.

Serial injections were made in the control state, in the empty beating heart state, during the course of ventricular fibrillation and coronary artery perfusion and one hour after coming off bypass.

In Group II, cardiopulmonary bypass was instituted but the aorta was cross-clamped for 60 minutes and continuous iced saline irrigation (10°C) of the heart performed while the heart was suspended in a pericardial cradle. As in the first group, moderate systemic hypothermia (30°C) was used.

Serial injections were made at a control state (pre-bypass). In 5 dogs, serial injections were made in the empty beating heart state before cross-clamping, and in one 10-15 min. after removing the cross-clamp with the heart spontaneously beating, and the last injection was made one hour after coming off the pump.

Results: Preliminary results show that even though Group I was maintained for about 40 minutes longer on the pump, the endocardial/epicardial ratio was significantly increased one hour after coming off the pump (.971 vs. 1.141) ($p < .001$).

On the other hand, Group II showed a significant reduction of the endocardial/epicardial ratio (1.126 vs. .834) ($p < .025$).

This data would indicate that aortic cross-clamping with topical hypothermia is less effective than coronary perfusion even with ventricular fibrillation in myocardial preservation during cardiopulmonary procedures.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02636-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Methylprednisolone - I. Its Effects on Normal Regional Myocardial and Renal Blood Flow - II. Its Effects on "Vulnerable" Myocardium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI

OTHER: Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI
Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Surgery

SECTION --

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	2/3	1/3

SUMMARY OF WORK (200 words or less - underline keywords)

Methylprednisolone sodium succinate has been recommended by some as beneficial in the treatment of low cardiac output and ischemic states. Many investigators have documented certain beneficial inotropic and chronotropic effects of this drug on the heart, including direct coronary artery vasodilation.

In the renal circulation, various shock states and various drugs redistribute blood flow from the outer cortex to the inner cortex where juxtamedullary glomeruli have a greater capacity of retaining Na^+. The technique of radioactive microspheres is used to determine if methylprednisolone is beneficial to normal dog hearts and kidney, and in addition to a region of the heart made "vulnerable" by reducing the mean distal left circumflex artery pressure to a level of around 20 mm. Hg.

1064

Project Description: Part I. Normal adult American foxhounds are instrumented to measure BP, HR, cardiac output, dp/dt, left circumflex artery flow, and LAP. Methylprednisolone (30 mg./Kg) is given in rapid infusion, resembling clinical use, through a catheter in the right femoral vein.

Microspheres are injected and hemodynamic measurements are taken at the following intervals: control - baseline, during SM infusion, 30 min. after SM infusion, and 60 min. after SM infusion.

Part II. Several dogs will be instrumented in the same manner except that instead of having a flow probe attached to their circumflex artery, a 20 gauge short intracath will be inserted in this artery and securely tied to the epicardial fat. A screw clamp will then be placed proximally and secured down until a distal circumflex pressure of around 70 mm. Hg is achieved.

Afterwards, the same protocol for infusion of solu-medrol and the microspheres will be allowed.

Results: Part I: Blood flow to all layers of the heart and kidneys increased significantly. In the myocardium there was significant redistribution of flow to endocardium during infusion of this drug ($p < .05$), in spite of statistically significant drop in mean blood pressure ($p < .005$). And even though the endocardial/epicardial ratio was reversed at 30 ($p < .0125$) and 60 minutes ($p < .005$) after infusion of the drug, the blood flow to both endocardial and epicardial layers were still significantly increased ($p < .0005$ for both).

Part II: In the vulnerable myocardium, methylprednisolone appears to cause a similar enhancement of blood flow to all layers without changing the endocardial/epicardial ratio significantly. Further work is in progress.

We hope these results would indicate that methylprednisolone may be of use in patients with ischemic cardiac disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02637-01 SU

PERIOD COVERED July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Effects of Prostaglandins A_1 and E_1 on Regional Myocardial Blood Flow

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI
Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI

OTHER: Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	½	¼

SUMMARY OF WORK (200 words or less - underline keywords)

PGA_1 and PGE_1 have been shown to have a direct vasodilatory effect on regional vascular beds. Some investigators believe they are the principal mediators of coronary vasodilatation. Both drugs have been reported to increase coronary blood flow, heart rate, cardiac output, and dp/dt while decreasing mean aortic pressure and peripheral vascular resistance. These alleged actions of prostaglandins on the heart has prompted us to study their effect on cardiac hemodynamics and regional myocardial blood flow.

1066

Description: American foxhounds are currently being used for determination of HR, BP, cardiac output, dp/dt, LAP, and left circumflex coronary flow in the control basal state, during PGA_1 or PGE_1 administration and 15 minutes after the infusion.

PGA_1 is administered intravenously in doses of 1 μgm/Kg/min for three minutes while PGE_1 allows the same dose and infusion time but is administered through the left atrium due to the fact that it is metabolized to a great extent in the lungs.

Labeled radioactive microspheres are used to determine regional myocardial blood flow. They are injected in the following order: during the control state, at the start (30 sec.) of the infusion, at the end (2 1/3 - 3 min.) of the infusion, and about 15 minutes after the infusion has been completed.

Results: This is an ongoing project and while no permanent or final data have been computed as of this moment, it appears that we are not reproducing the findings reported previously in the literature. Both drugs seem to exert a significant negative inotropic effect on the heart. Further work is in progress.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02638-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Unrestrictive and Restrictive Reactive Hyperemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI

OTHER: Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI
Andrew G. Morrow, Chief, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	½	½

SUMMARY OF WORK (200 words or less - underline keywords)

Reactive hyperemia is a normal physiologic event in response to an abrupt total obstruction of blood flow. In the myocardium this event has been noted for years but only recently has the technique been developed that allows the time related transmural blood flow pattern to this insult to be documented. Two group of dogs were studied using radioactive microspheres to determine the transmural myocardial blood flow during reactive hyperemia. The unrestrictive group, group I, underwent acute occlusion of the circumflex coronary artery for 15 seconds and transmural flow was determined at 15 seconds, forty-five seconds, and 90 seconds after release of the occlusion. The restrictive group, group II, was the same as group I, but in addition had a partial occlusion of the circumflex coronary artery that maintained the distal coronary pressure at approximately 80 mm. Hg mean pressure. Transmural blood flow pattern of group I revealed that both epicardium and endocardium underwent reactive hyperemia of identical magnitude and that the time of peak flow in each was identical. Furthermore, the endocardial/epicardial flow ratio remained normal throughout the reactive hyperemic response. Preliminary data from group II would indicate that the epicardial flow is greater in magnitude and earlier in time than that of the endocardium which is of lesser magnitude and later in time in the reactive hyperemia response.

1068

PHS-6040
(12-75)

Description: Reactive hyperemia is a normal physiologic event in response to increased oxygen demand over oxygen supply in the myocardium. This study was designed to determine the tissue-related transmural myocardial blood flow in unrestrictive hyperemia and as it compares to restrictive hyperemia.

Two groups of adult American foxhounds were used in this acute study. The animals after being anesthetized and maintained on a mechanical ventilator underwent a left thoracotomy and pericardiotomy for exposure. The left circumflex coronary artery was dissected free for approximately 2 cm. near its origin and the appropriately sized electromagnetic flow probe was implanted. A left atrial line was inserted through the left atrial appendage for injection of radioactive microspheres. An arterial line was established in each femoral artery for reference grouping of the microsphere technique, and monitoring blood pressure. Then an acute 15 cc. total occlusion of the coronary artery was done in the unrestrictive group, Group I. In the restrictive group, Group II, there was in addition an adjustable device used to develop a critical stenosis of the coronary artery just proximal to the flow probe. Distal coronary pressure was monitored by an intra-arterial line and was maintained above 80 mm. Hg by the adjustable device used to produce the stenosis.

Radioactive microspheres were used to determine surgical myocardial blood flow in the resting state, and 15 sec., 45 sec., and 90 sec. after release of the occlusion. By using the reference sampling technique, blood flow can be calculated in ml/gm/min.

Results: Transmural blood flow in Group I revealed that not only did both the epicardium and endocardium undergo a reactive hyperemia response but each respectively developed a response which was identical in magnitude and elapsed time at peak flow. Thus, the endocardial/epicardial flow ratio remained normal throughout the unrestrictive hyperemic response.

The transmural blood flow during the restrictive hyperemia was different. Preliminary data would indicate that the epicardial flow was greater in magnitude, that the peak flow was earlier in elapsed time from occlusion and that the duration of response was shorter than that of the endocardium. In other words, the epicardial flow pattern of Group II was similar to that of Group I, while that of the endocardium was dissimilar. The endocardial pattern of Group II revealed the magnitude and peak flow were less while elapsed time at peak flow and duration of response were longer than that of Group I. Furthermore, the endocardial/epicardial flow ratio was varied. Earlier in the response, during maximal epicardial flow, the ratio was reduced and later in the response, during maximal endocardial flow, the ratio was increased. Transmural myocardial blood flow was determined in unrestrictive and restrictive reactive hyperemias. The data would support the argument that with a significant coronary artery stenosis, endocardial flow and oxygen demand are established secondary to endocardial flow and oxygen demand.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02639-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Myocardial Reflow Phenomena With and Without a Membrane Stabilizer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glenn E. Newman, Surgeon, Clinic of Surgery, NHLI
OTHER: Alberto J. Larrieu, Guest Worker, Clinic of Surgery, NHLI
James H. McClenathan, Surgeon, Clinic of Surgery, NHLI
Lawrence L. Michaelis, Senior Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1½	1	½

SUMMARY OF WORK (200 words or less - underline keywords)

Alteration or reversal of the cyclic and progressive results of myocardial ischemia is a dilemma in myocardial pathophysiology. This study attempts to quantitate regional and transmural patterns in myocardial blood flow in the anoxic working heart with and without the benefit of a membrane stabilizer - methylprednisolone as used in this study. Regional and transmural myocardial blood flow patterns were determined in four groups of dogs before LAD coronary artery occlusion and after removal of the LAD occlusion. Groups I and II were controls having a one hour and two hour occlusion respectively before reflow patterns were determined. Groups III and IV were studied similarly, except that methylprednisolone was given one time in a pharmacologic dose prior to the LAD occlusion in each animal. Patterns of regional and transmural myocardial blood flow were determined by the radioactive microsphere technique and a vital dye - thioflavin-S. Group I reflow pattern was that of progressively decreased flow from epicardium to endocardium, even though the total transmural flow was normal. Group II was worsened. The total transmural flow was decreased, and the transmural maldistribution of Group I was further accentuated. Furthermore, the total size of the ischemic area was increased. Group III reflow pattern was almost normal regionally and transmurally, indicative of only reversible metabolic events. Group IV reflow was similar to that of Groups I and II.

1070

PHS-6040
(12-75)

Description: The pathophysiology of the cyclic and progressive events of prolonged myocardial ischemia is a dilemma in current research. Furthermore, methods of favorably altering these events are at best poorly understood. However, this study was designed to determine transmural patterns of myocardial blood flow in the regionally anoxic working heart with and without the benefit of a membrane stabilizer.

Regional and transmural myocardial blood flow patterns were determined in four groups of adult American foxhounds. Each dog underwent a left thoracotomy after anesthesia and ventilatory support. The left anterior descending coronary artery was dissected free for approximately 2 cm. near its origin and an electromagnetic flow probe and snare was placed in position. A left atrial catheter was put through the left atrial appendage for injection of microspheres. Each femoral artery was used to monitor blood pressure and withdraw reference samples. Groups I and II underwent a one hour and two hour LAD occlusion respectively before reflow patterns were determined sequentially at 5 minutes and 30 minutes after release of the occlusion. Groups III and IV were studied similarly except that methylprednisolone was given in one pharmacologic dose prior to the LAD occlusion in each animal. After the last injection of microspheres, thioflavin-S, a vital dye, was injected into each animal and then each animal was sacrificed. The heart was removed and studied under a fluorescent lamp to separate the region perfused by the LAD from the remainder of the heart. Generally, that region perfused by the circumflex artery was used as a control.

An injury weight index was determined by dividing the weight of the injured area by the weight of the total left ventricular free wall. The radioactive microsphere technique was used to determine the transmural blood flow patterns sequentially in each arrival. This technique allows quantitative determinations of flow to tissue in terms of ml/gm/min . Four data points were obtained in each arrival: a control point, a 4 min. pre-release occlusion point, a 5 min. post-release occlusion point, and a 30 min. post-release occlusion point.

Results: Preliminary data suggest that methylprednisolone favorably alters the progressive events of regional myocardial ischemia. In Group I the reflow pattern was that of progressively decreased flow from epicardium to endocardium and a marked reduction of the endocardial/epicardial ratio. However, the total transmural flow was normal. In Group II, the total transmural flow was decreased and the transmural maldistribution pattern of Group I was further accentuated. Furthermore, the injury weight index increased.

Groups III and IV had been given methylprednisolone before the LAD occlusion. In Group III, the transmural flow was normal or increased. In some animals, the epicardium showed a reactive hyperemic response at 5 minutes after release of the occlusion. More importantly, the endocardium was well maintained in terms of blood flow.

In Group IV, the transmural flow was subnormal but not markedly reduced. The epicardial flow was normal but the endocardial flow was less well maintained. Thus the endocardial/epicardial ratio was reduced slightly.

Furthermore, the injury weight indices of Groups III and IV were less than Groups I and II respectively.

The preliminary data suggest that the events following prolonged regional myocardial anoxia are progressive and eventually irreversible within a reflow period of 30 minutes. Methylprednisolone can substantially delay the progressive nature of these events. In other words, methylprednisolone can substantially prolong the reversible injury time and therefore can favorably alter the pathophysiological events of prolonged regional myocardial ischemia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02640-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Electrocardiographic Determination of Left Ventricular Size Following Cardiopulmonary Bypass

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Vicent A. Gaudiani, Surgeon, Clinic of Surgery, NHLI
Walter L. Henry, Senior Investigator, Cardiology Branch, NHLI

OTHER: Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
James M. Griffith, Electronics Engineer, BEI
Andrew G. Morrow, Chief, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	½	¼

SUMMARY OF WORK (200 words or less - underline keywords)

An echocardiographic probe has been designed to be sutured to the left ventricle during operation and to be removed percutaneously after several days. This device monitors changes in left ventricular systolic and diastolic dimensions during, and after any cardiac surgical procedure. The object is to provide direct measurement of left ventricular function following operation, correlate measurement with current monitoring techniques, and determine the effect of postoperative interventions on cardiac performance.

1073

Description:

The probe is composed of an eliptical 2.5 mhz echo crystal embedded in a lozenge shaped plexiglass container which is 1" x 1/4" x 3/8". Four holes have been drilled through the edges to allow placement of 4-0 chromic sutures. The intrathoracic portion of the probe is coated with a nonreactive polymer. The device is sutured to the left ventricular epicardium and its connecting wire is brought out through a small incision in the skin. Accurate placement is assured by monitoring the echocardiogram obtained while placing the crystal on the ventricular wall. The crystal is placed to record septal motion, maximum left ventricular dimension, and free wall motion. It may be placed during any procedure in which the heart is exposed through median sternotomy or left thoractomy.

Results:

The device has been sutured to the left ventricle through a left thoracotomy in five dogs for periods of two to five days. Several observations show that reproducible determinations of LV size can be made. All the dogs survived removal of the probe percutaneously, under light sedation without serious arrhythmias. At postmortem, none of the hearts showed injury caused by the probe. The device will be placed on the left ventricle in a group of patients undergoing mitral and aortic valve replacement.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02641-01 SU

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Anaerobic Cardiac Perfusion During Cardiopulmonary Bypass with Aortic Cross-Clamp

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLI

OTHER: Kenneth M. Kent, Head Sec. Exp. Physiology, Cardiology Branch, NHLI
Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLI
David G. Kastl, Surgeon, Clinic of Surgery, NHLI

COOPERATING UNITS (if any)
Cardiology Branch

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	2/3	1/3

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to determine if low flow, buffered, asanguinous solutions (Krebs-bicarbonate buffer with glucose) can prevent ischemic myocardial damage during cardiopulmonary bypass with aortic cross-clamp. Recent work suggests that intracellular acidosis initiates the sequence of events which lead to ischemic damage. In addition, energy production by anaerobic pathways may be sufficient to support the nonworking heart. Providing this solution to the coronary bed may prevent acidosis and promote glycolysis.

1075

Description: Ten dogs have been placed on cardiopulmonary bypass at 30°C. In Group I no further intervention was made. In the other two groups, aortic cross-clamp was applied for one hour. In Group II, no other intervention was made. In Group III, the aortic root was perfused with Krebs-bicarbonate buffer containing 250 mg.% of glucose at 30° at a rate of 25 cc./min. In all three groups, ventricular function curves were obtained before and after bypass. All Group I dogs showed identical function curves before and after the procedure. All Group II dogs showed severely depressed function after one hour of cross-clamp. All Group III dogs showed no depression of function using the Krebs buffer perfusate. These preliminary results suggest that low flow buffered perfusates afford myocardial protection.

The numbers of dogs in each group must be enlarged to confirm early observations. Coronary sinus lactates must be obtained, and the limit of cross-clamp time for this type of performance must be ascertained.

Annual Report of the Section of Pathology

Office of the Director

of the Division of Intramural Research

National Heart and Lung Institute

July 1, 1975 through June 30, 1976

This section is concerned primarily with the study of structural alterations produced by various cardiovascular and pulmonary diseases. Structural alterations are studied at gross, light microscopic, ultrastructural and histochemical levels. Studies during this period focused on coronary, valvular, congenital, and myocardial heart diseases.

CORONARY HEART DISEASE

A continuing major undertaking of this laboratory is the examination in more or less serial fashion of the coronary arterial tree and a number of different conditions. Previous studies in this laboratory have focused primarily on the status of these arteries in patients with fatal acute transmural myocardial infarction and in patients with sudden coronary death. Studies during the period covered by this report have focused on:

a) The coronary arteries and left ventricular myocardium in angina pectoris. Until coronary bypass procedures came into existence, it was not possible to study the coronary arteries or myocardium in patients with clinically isolated angina pectoris because most of these patients died from either acute myocardial infarction or sudden coronary death and therefore were classified in those categories. A study of 27 patients who died following aorto-coronary bypass procedures disclosed extreme degrees of severe coronary atherosclerosis in each. The lumens of at least two (avg, 3.0) of the 4 major epicardial coronary arteries were narrowed >75% in cross-section of area by old atherosclerotic plaques in these patients. Despite the severe coronary narrowing, however, there was little myocardial damage. Left ventricular scarring, for example, was observed grossly in only 14 (52%) of the 27 patients and in each it involved only a small portion of left ventricular myocardium. Furthermore, the left ventricular cavity was of normal size except in 2 patients, and the heart was of normal weight in 56% of the patients and the average increase above the upper range of normal for all 27 patients was only 5%. Thus, clinically isolated severe angina pectoris is associated with severe diffuse luminal narrowing but relatively little myocardial damage. This was the first study of coronary arteries of patients with clinically isolated angina pectoris.

b) Atherosclerotic narrowing of the left main coronary artery. In 1972 it was pointed out by coronary angiographic studies that patients with severe narrowing of the left main coronary artery often had a poor prognosis and high risk to cardiac catheterization and coronary bypass operation. A morphologic study of these patients has not been done. Therefore, a group of 154 necropsy patients with fatal ischemic heart disease was studied. It was found that angina pectoris and type II hyperlipoproteinemia were more common, and that acute transmural and healed subendocardial myocardial infarction were less frequent, in the patients with >75% left main narrowing than in those with <50% narrowing. Of the 3 other major coronary arteries, the average number of vessels narrowed was 2.4 in the patients with <50% left main narrowing and 2.9

1077

in the patients with >75% narrowing. Narrowing of the left main coronary artery, therefore, is indicative of severe narrowing of all four major coronary arteries.

c) Angiographically normal coronary arteries after healing of acute myocardial infarction. There have been a number of reports in recent years of patients with "angiographically normal coronary arteries and myocardial infarction." This study examined patients who had had transmural acute myocardial infarction and at necropsy had been found to have near normal coronary arteries, or at least <75% narrowing of these vessels; in addition, it reviewed the reports of all reported patients with angiographically normal coronary arteries and myocardial infarction. It was apparent that there has never been a report of angiographically normal coronary arteries at the time of acute myocardial infarction. The coronary angiography has been done in all cases at a later period; the earliest reported is one month. Six possible explanations for the occurrence of angiographically normal coronary arteries and myocardial infarction were proposed and evidence was presented that coronary embolism with subsequent lysis or recanalization was the most likely reason for this occurrence.

d) Coronary heart disease after mediastinal irradiation. Two young adults were studied who had developed fatal ischemic heart disease after heavy administration of irradiation for Hodgkin's disease. It was demonstrated that if given sufficient amounts, radiation can cause severe damage to the coronary arteries with fatal consequences.

e) Tension on coronary bypass conduits, a cause of obstruction in saphenous vein grafts. Abnormal tension on saphenous vein-aorto-coronary bypass grafts were found at necropsy in 16 of 41 grafts. The abnormal tension resulted from insertion of grafts that were too short to accommodate normal or abnormal distension which the right atrium, right ventricle or pulmonary trunk undergo after discontinuation of cardiopulmonary bypass or development of acute congestive cardiac failure. It was apparent that some of these grafts needed to be longer at the time of insertion.

f) Heterografts as aorto-coronary bypass conduits in human beings. One patient was studied who had had insertion of sheep carotid arterial heterografts and a canine saphenous vein heterograft. It was apparent from study of this patient and review of others that heterografts are unsatisfactory as aorto-coronary bypass conduits.

VALVULAR HEART DISEASE

A continuing activity of this section has been the study of patients with valvular heart disease, to gain more information regarding the natural history of various valvular lesions and the complications of prosthetic valves. Many reports are available regarding hemodynamic evaluation of various prosthetic valves but, in contrast, relatively few have appeared evaluating these valves from a morphologic standpoint. Previous studies from this laboratory have focused primarily on caged-ball, caged-disc prostheses and some tissue valves. During this past year, clinical and necropsy observations in a large group of patients who had died after insertion of one or more tilting disc prostheses of the Björk-Shiley type were compared to those made earlier in patients with caged-ball, caged-disc and trileaflet prostheses. Although it is prone to clot and therefore requires long-term anticoagulation, the Björk-Shiley tilting disc prosthesis is not intrinsically stenotic or significantly incompetent; it is only minimally traumatic to erythrocytes and, at this time, there is no evidence of prosthetic degeneration or variance. Therefore, it is a good substitute cardiac valve.

One of the worst complications of prosthetic valve replacement is prosthetic valve endocarditis. Observations in a group of 23 patients with fatal prosthetic valve endocarditis was reviewed and compared to those in 74 patients with active infective endocarditis involving natural left-sided cardiac valves. Comparison between the 2 groups showed significant differences in incidence of: ring abscess, hemodynamic consequences of the endocarditis (valve stenosis), Staphylococcus being the causative organism, and complete heart block or left bundle branch block. No significant differences were observed between the 2 groups when comparing age, sex, type of underlying valve disease, or frequency of organ infarcts or splenomegaly.

A review was made of the experience of the Section of Pathology with patients with active infective endocarditis involving natural valves. This review included: 23 patients with infective endocarditis involving right-sided cardiac valves, with or without involvement of left-sided valves; 74 patients with infective endocarditis limited to left-sided cardiac valves; 4 patients with infective endocarditis after valvuloplasty operations, and 6 patients with infective endocarditis complicating congenital heart disease with a shunt. From the experience of the past 15 years, it is clear that infective endocarditis is considerably more common on previously normal cardiac valves, i.e., normal both anatomically and functionally, than on previously abnormal valves.

A special study was made of the frequency, location and clues to clinical diagnosis of valve ring abscess in active infective endocarditis. Of 95 necropsy patients with active infective endocarditis involving 128 native valves, 27 had ring abscess involving 30 valves. Comparison of the following parameters showed significant differences between the 27 patients with and the 68 patients without valve ring abscess: 1) infection of the aortic valve; 2) occurrence of valvular regurgitation of recent origin; 3) presence of pericarditis; 4) presence of high degree of atrioventricular block, and 5) short duration of symptoms leading to severe debility or death.

The ideal characteristics of a substitute cardiac valve were analyzed in terms of the 4 basic types of presently available substitute cardiac valves. Of the 4, it is clear that the caged-disc prosthesis is the <u>least</u> desirable. Ideally, the caged-ball prosthesis might be limited to those patients with volume lesions, i.e., pure or at least predominant regurgitation, because of the problem of prosthetic disproportion. The tilting disc has favorable dynamic and wear characteristics. Long term anticoagulants are essential as with the caged-ball and caged-disc type of prostheses. The porcine xenograft at this time appears quite favorable but sufficient, i.e., >5 years, implantation periods for proper evaluation are just beginning to occur and, therefore, final judgment must be reserved on this type of valve prosthesis.

For many years, many books and articles have spoken of a functional cause of mitral regurgitation, the so-called "relative mitral regurgitation" due to dilatation of the mitral anulus. To confirm or deny this thesis that mitral anular dilatation is a cause of mitral regurgitation, this anulus was measured in 102 patients, 24 of whom had normal hearts and 78 of whom had dilated left ventricles. Some of these patients had mitral regurgitation; others did not. It was clear that dilatation of the mitral anulus was a rare cause of mitral regurgitation and that abnormality of the fibrous skeleton of the heart is necessary for the mitral anulus to dilate enough to cause mitral regurgitation. The anulus must dilate >50% over normal for regurgitation to result from this means alone.

CONGENITAL HEART DISEASE

Although a relatively uncommon lesion, aortic valve atresia is an important congenital cardiovascular malformation because it represents the least tolerated cardiac anomaly, and therefore one extreme of the spectrum of congenital heart disease. The hearts of 73 patients with fatal aortic valve atresia were examined and a new classification of this entity was presented. Although 69 of the 73 patients had extremely hypoplastic left ventricles, four had normal or nearly normal-sized left ventricles because a ventricular septal defect was present. Although operative intervention in patients with hypoplastic left ventricles associated with aortic valve atresia is fruitless, it appears that it may be fruitful in individuals with normal or near normal-sized left ventricular cavities.

Prolapse of the mitral valve is an entity which has received considerable attention for the past 15 years. Two patients were studied in whom the classic Barlow's syndrome was present in association with Ebstein's anomaly of the tricuspid valve. This association had not been described previously.

During the past 16 years there has been an opportunity to study, at necropsy, a number of patients with pulmonary venous hypertension of congenital origin. A clinical and morphologic review of the causes of congenital pulmonary venous hypertension was undertaken.

PERICARDIAL HEART DISEASE

Pericardial heart disease is one of the less common of the major types of cardiac disease. A large review was undertaken this year of all patients in whom pericardial heart disease was observed at necropsy during the past 15 years.

PULMONARY HEART DISEASE

A continuing colaboration is going on between the Section of Pathology and the Pulmonary Disease Branch of NHLI. Focus has been on idiopathic pulmonary fibrosis and a major morphologic paper on this subject is presently in preparation.

MYOCARDIAL DISEASES

As part of our continuing program on the ultrastructural pathology of myocardial diseases, studies were made of lesions in the Hurler syndrome, selenium-vitamin E deficiency, and infantile cardiomyopathy with histiocytoid change (a clinicopathology syndrome that was fully defined for the first time). In addition, a study was also made of cardiac findings in patients undergoing bone marrow transplantation.

Hurler syndrome: The coronary arteries, the 4 cardiac valves, mural endocardium of all chambers, the myocardial walls and the aorta were affected characteristically in 5 necropsy patients with the Hurler syndrome. These sites contained large, clear cells (Hurler cells) filled with deposits of acid mucopolysaccharides (AMP). The coronary arteries, atrioventricular valves and myocardial interstitium also contained granular cells, some of which had intracellular collagen deposits. Deposits of AMP and glycolipids also were present in cardiac muscle cells, smooth muscle cells and fibroblasts. These changes resulted in severe narrowing of extramural coronary arteries, considerable thickening of valves and mural endocardium, and stiffening of cardiac walls. Thus, the cardiovascular lesions in the Hurler syndrome are specific and life-threatening.

Selenium-vitamin E deficiency: Studies of the ultrastructure of skeletal and cardiac muscle of animals with experimentally induced selenium-vitamin E deficiency (a disease that occurs spontaneously in sheep, cattle, pigs and poultry and is of considerable economic importance to farmers) were made in collaboration with Dr. John F. Van Vleet and his associates at Purdue University. Studies of skeletal muscle showed similar changes in pigs and chicks, with the earlies changes being myofibrillar lysis, followed by damage to mitochondria, sarcoplasmic reticulum and plasma membranes. The basal lamina of the sarcolemma served as a scaffold for subsequent muscle regeneration. Studies of cardiac muscle in this disease are in progress.

Infantile cardiomyopathy: Clinical and pathologic findings were reported in 6 patients, all children ranging in age from 6 to 24 months, with a clinicopathologic syndrome termed "infantile cardiomyopathy with histiocytoid change in cardiac muscle cells". This syndrome is manifested by 1) fatal cardiac arrhythmias; 2) cardiac hypertrophy, and 3) focal degeneration of the muscle cells, which lose their myofibrils, undergo mitochondrial hyperplasia, become rounded and enlarged, and resemble histiocytes. It is concluded that these manifestations are those of a cardiomyopathy, that hypertrophy precedes the onset of the clinical features, that the focal degeneration is likely to cause the arrhythmias, and that the latter develop only late in the disorder. The etiology of this cardiomyopathy remains unclear.

Cardiac findings in patients with bone marrow transplants: Cardiac pathologic findings were analyzed in 20 necropsied patients with leukemia, aplastic anemia, immune deficiency disease or metastatic cancer who had been treated with bone marrow transplantation (BMT). Some findings resembled those in patients with hematologic and neoplastic diseases who had not been treated with BMT. A distinctive interstitial reactive change (characterized by lymphoid, histiocytic and Anitschkow cells) was considered to have been induced by abnormal immune mechanisms related to BMT. Two patients developed fatal cardiac failure after BMT and exhibited severe, acute myocardial injury. The latter resulted primarily from high doses of cyclophosphamide which were administered as part of a newly developed regimen of combination chemotherapy-immunosuppression.

CARDIAC HYPERTROPHY

It has become evident from a number of studies that the ultrastructural features of hypertrophied hearts vary according to the cause and the stage of the hypertrophy, that different organelles of cardiac muscle cells behave differently (both qualitatively and quantitatively) in response to the stimuli of hypertrophy, and that the late stages of hypertrophy are characterized by degeneration of cardiac muscle cells. As part of our long-range study of cardiac hypertrophy, we have undertaken a series of detailed morphologic investigations of changes that individual cellular components undergo during hypertrophy. We have previously described alterations which occur in hypertrophy and involve nuclei and nuclear membranes, intercellular junctions, myofibrils, mitochondria and cytoskeletal filaments. This year, studies were made of alterations involving sarcolemma, sarcoplasmic reticulum, and the compartmentalization of glycogen particles in cardiac mucle cells.

Sarcolemma: Studies of alterations in the sarcolemma were made in tissues obtained from over 100 patients with cardiac hypertrophy of various causes, from dogs subjected to anoxic cardiac arrest under total cardiopulmonary bypass,

and from rabbits given large doses of anthracyclines (antineoplastic agents with cardiotoxic properties). Sarcolemmal changes in hypertrophied, nondegenerated cardiac muscle cells consisted of: increase in area and degree of irregularity of cell surfaces; dilatation of T-tubules, and formation of numerous multiple intercalated discs. Sarcolemmal alterations in cells with severe degeneration were associated with myofibrillar loss and consisted of: disappearance of T-tubules; loss of intercellular junctions; marked surface irregularities; formation of intracytoplasmic junctions, and thickening of basement membranes. These observations show that remodeling of the surfaces of cardiac muscle cells is an important part of the cellular response to the stimuli of hypertrophy or degeneration.

Sarcoplasmic reticulum (SR) in atrial myocardium: Important and extensive morphologic changes were found to occur in SR of atrial myocardium from 10 patients with mitral valvular disease. These changes consisted of: 1) proliferation of rough-surfaced endoplasmic reticulum, a change indicative of increased protein synthesis; 2) proliferation of free SR reticulum, a change related to loss of contractile elements; 3) two types of aggregates of tubules of free SR: one type was associated with abnormal Z-band material and loss of myofibrils, whereas the other occurred in less severely altered cells; and 4) proliferation and enlargement of cisterns of extended junctional SR. This is the first detailed study made of SR in human atrial myocardium.

Intranuclear glycogen: Glycogen deposits were found within nuclei of cardiac muscle cells in 6 of 90 patients with cardiac diseases of various causes. It was concluded that under some circumstances the enzymes involved in glycogen synthesis can become translocated (presumably in relation to increased permeability of nuclear membranes) from the cytoplasm to the nucleus, where they eventually synthesize glycogen, and that such a phenomenon is not necessarilly indicative of damage or degeneration.

CARDIAC TUMORS

Cardiac rhabdomyomas: In previous studies of cardiac tumors we have described the ultrastructure of myxomas and papillary endocardial elastofibromas. This year an extensive study was made of cardiac rhabdomyomas. Of cardiac rhabdomyomas in 36 patients, 90% were multiple and 78% occurred in children under 1 year of age. In 56% of the patients at least one of the tumors was intracavitary and in 42% it obstructed 1 or more valves. The tumors occurred with equal frequency in the right and left ventricles and in subepicardial and subendocardial locations. Ultrastructural study of tumors from 7 patients revealed: paucity of myofibrils, which were arranged either parallel to the cell surfaces or in strands radiating toward the center; randomly located intercellular junctions (which resembled intercalated discs of normal myocardium) and numerous leptofibrils. Cardiac rhabdomyomas are derived from muscle cells and probably represent hamartomas rather than true tumors.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03001-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Coronary Arteries in Ischemic Heart Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. C. Roberts Chief, Sect. Path. NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This article summarizes observations on coronary arteries in fatal ischemic heart disease and also summarizes observations on acute or recent lesions particularly in these patients.

1083

PHS-6040
(12-75)

Project No. Z01 HL 03001-02 OD P

Project Description: This article summarizes observations on coronary arteries in fatal ischemic heart disease and also summarizes observations on acute or recent lesions particularly in these patients: 1) among patients with fatal ischemic heart disease, thrombi are infrequent in patients dying suddenly and in those in whom the necrosis was limited to subendocardium; 2) thrombus is found in a coronary artery in about 60% of patients with fatal transmural acute myocardial infarction; 3) among patients with transmural myocardial necrosis, the major determinant of the presence of coronary thrombosis appears to be cardiogenic shock; 4) the larger the area of myocardial necrosis the greater the likelihood of coronary thrombosis; 5) when coronary thrombosis is associated with acute myocardial infarction, the thrombus is always located in the artery responsible for profusing the area of myocardial necrosis; 6) thrombi occur in fatal ischemic heart disease in coronary arteries which are already severely narrowed by old atherosclerotic plaques; 7) coronary thrombi in fatal acute myocardial infarction are usually occlusive, short, and located entirely in the major trunks.

Publications: Roberts, W.C.: The Status of the Coronary Arteries in Fatal Ischemic Heart Disease. In Brest, A.N., Wiener, L., Chung, E.K., Kasparian, H. (Eds.): Inovations in the Diagnosis and Management of Acute Myocardial Infarction. Philadelphia, F.A. Davis, 1975, pp. 1-20.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03002-02 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Coronary Arteries in Fatal Coronary Events

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W.C. Roberts Chief, Sect. Pathol. NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report emphasizes certain aspects of the coronary tree in patients with various types of fatal coronary events. Points emphasized are the role of coronary thrombosis in acute myocardial infarction.

1085

PHS-6040
(12-75)

Project Description: This report emphasizes certain aspects of the coronary tree in patients with various types of fatal coronary events. Points emphasized are the role of coronary thrombosis in acute myocardial infarction. Two factors implicate coronary thrombosis as the precipitating cause of acute myocardial infarction: 1) the occurrence of coronary arterial thrombi in many patients with fatal acute myocardial infarction and 2) the location of the thrombus in the coronary arteries responsible for supplying the area of myocardial necrosis. Five factors, however, tend to indicate that coronary thrombosis is a consequence rather than the precipitating cause of acute myocardial infarction: 1) the low frequency of thrombi in patients dying suddenly with or without previous evidence of cardiac disease; 2) the increasing frequency of thrombi with increasing intervals between the onset of symptoms of acute myocardial infarction and death; 3) the absence of thrombi in fatal transmural acute myocardial infarction nearly as often as they are present; 4) the near absence of thrombi in fatal subendocardial infarction; 5) the occurrence of thrombi in high percentage only in patients with cardiogenic shock, most of whom have large transmural infarcts. It appears from study of 107 patients with fatal acute myocardial infarction in this laboratory that the key to coronary thrombosis just as the key to thrombosis occurring anywhere in the body is slow blood flow or relative statis and sufficient time for the thrombus to form. The absence of these 2 factors may explain the absence of coronary thrombosis in the sudden coronary death cases and the increasing frequency of thrombosis as the interval from onset of symptoms of myocardial ischemia to death increases.

Publication: Roberts, W.C.: The Coronary Arteries in Fatal Coronary Events. In Chung, E.K.: Controversy In Cardiology. New York, Springer-Verlag, 1976, pp. 1-22.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03003-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Coronary Arteries in Coronary Heart Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. C. Roberts Chief, Sect. Pathol. NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report summarized certain characteristic changes in the coronary arteries observed at necropsy in patients with fatal ischemic heart disease.

1087

PHS-6040
(12-75)

Project Description: This report summarizes certain characteristic changes in the coronary arteries observed at necropsy in patients with fatal ischemic heart disease: 1) the coronary arteries are diffusely involved by atherosclerotic plaques; 2) with rare exception, at least 2 of the 3 major coronary arteries are narrowed >75% by old atherosclerotic plaque; 3) the atherosclerotic process is limited to the epicardial coronary arteries; 4) certain portions of the coronary tree tend to develop larger atherosclerotic plaques and, therefore, more narrowed lumens than other portions; 5) of the so-called 3 types of atherosclertoic plaques, namely lipid, fibrous, complicated, only the complicated plaque is responsible for causing significant (>75%) narrowing of the lumens of the coronary arteries; 6) the degree of coronary arterial luminal narrowing by atherosclerotic plaques and the extensiveness of the plaquing are similar in patients with fatal ischemic heart disease irrespective of the type of fatal coronary event; 7) the composition of coronary atherosclerotic plaques and the degree of coronary arterial luminal narrowing in patients with fatal ischemic heart disease appear similar irrespective of whether or not the blood lipoprotein pattern is normal or abnormal; 8) the shapes of lumens of atherosclerotic coronary arteries are quite variable; 9) and, although the number 1 risk factor to development of atherosclerosis in the western world, advanced age does not necessarily indicate the presence of severe coronary atherosclerosis.

Publications: Roberts, W. C.: The Coronary Arteries in Coronary Heart Disease: Morphologic Observations. In Ioachim, H. L. (Ed.): Pathobiology Annual 1975. New York, Appleton-Century-Crofts, 1975, pp. 249-282.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03006-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Thrombosis, Atherosclerosis and Ischemic Heart Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol. NHLI
OTHER:	V. J. Ferrans	Senior Investigator, Sect. Pathol. NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report summarizes observations learned during the past couple of years in this laboratory suggesting that atherosclerotic plaques result, at least in part, from organization of thrombi.

1099

PHS-6040
(12-75)

Project Description: This report summarizes observations learned during the past couple of years in this laboratory suggesting that atherosclerotic plaques result, at least in part, from organization of thrombi: 1) the presence of known components of thrombi--namely fibrin and platelets--within atherosclerotic plaques; 2) the occurrence of known atherosclerotic plaques--namely foam cells, cholesterol clefts, pultaceous debris, calcium--in organized hematomas or known thrombi wherever they occur in the body; 3) the presence of multiluminal channels in vessels, a recognized consequence of organization of pulmonary thromboemboli--and presumably also coronary thrombi or emboli; 4) the major component of the complicated atherosclerotic plaque, i.e., that capable of causing significant luminal narrowing, in the coronary arteries of patients with fatal ischemic heart disease is fibrous tissue or collagen, not lipid and this is true whether or not hyperlipidemia is or was present; 5) experimentally induced thrombi under proper conditions may be transformed into atherosclerotic plaques closely resembling those observed in human coronary arteries. The above factors do not prove that thrombosis is the cause of atherosclerosis, but together they strongly suggest that organization of thrombi plays a major role in the development of the complicated atherosclerotic plaque.

Publications: Roberts, W. C. and Ferrans V. J.: The Role of Thrombosis in the Etiology of Atherosclerosis (A Positive One) and in Precipitating Fatal Ischemic Heart Disease (A Negative One). In Donoso, E. and Haft, J.I. (Eds.): Thrombosis, Platelets, Anticoagulation, and Acetylsalicylic Acid. New York, Stratton Intercontinental Medical Book Corporation, 1976, pp. 143-155.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03007-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Acute Myocardial Infarction and Angiographically Normal Coronary Arteries

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. N. Arnett	Staff Associate, Sect. Pathol.	NHLI
OTHER:	W. C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report examines previous reports of myocardial infarction and angiographically normal coronary arteries and discusses possible explanations for the occurrence of "myocardial infarction and normal coronary arteriograms." Five explanations are considered and evidence is presented that the acute myocardial infarction was caused by an occluding embolus which subsequently lysed or recanalized.

1091

PHS-6040
(12-75)

Project Description: This report examines previous reports of myocardial infarction and angiographically normal coronary arteries and discusses possible explanations for the occurrence of "myocardial infarction and normal coronary arteriograms." There have been 45 reported patients with "myocardial infarction and angiographically normal coronary arteries:" in 5 the acute myocardial infarction was produced at the time of cardiac catheterization and in the other 40 patients acute myocardial infarction was unrelated to cardiac catheterization. In 39 of the 40 patients with non-catheter related infarction the interval between the onset of acute myocardial infarction and performance of coronary angiography was longer than 2 months. Thus, the status of the coronary arteries at the time when a portion of left ventricular myocardium was noted is uncertain. Indeed, a normal coronary arterial tree has never been demonstrated by angiography at the time of acute myocardial infarction. This report examines then how a coronary tree may be entirely normal by angiography after healing of an acute myocardial infarction. Five explanations are considered and evidence is presented that the acute myocardial infarction was caused by an occluding embolus which subsequently lysed or recanalized.

Publications: Arnett, E.N. and Roberts, W.C.: Acute Myocardial Infarction and Angiographically Normal Coronary Arteries. An Unproven Combination. Circulation 53:395-400, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03008-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Myocardial Embolus to Coronary Artery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. J. Hammer	Cardiology Fellow, Div. Cardiol. Dept. of Med. Georgetown Univ., Washington, D.C.	
OTHER:	V. J. Ferrans	Senior Investigator, Sect. Pathol.	NHLI
	W. C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
Division of Cardiolovy, Department of Medicine, Georgetwon University, Washington, D.C.

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hours	175 hours	0

SUMMARY OF WORK (200 words or less - underline keywords)

Of over 200 hearts examined systematically at necropsy in patients with fatal coronary heart disease, one was observed to have an embolus of necrotic myocardium in a coronary artery. Necropsy disclosed rupture of one left ventricular papillary muscle and a clump of myocardium in the lumen of the right coronary artery.

1093

PHS-6040
(12-75)

Project Description: Although infrequent, emboli to coronary arteries usually consist of fibrin and platelets. Other dislodged material in coronary arteries have included calcific debris, clumps of neoplastic cells, suture and other foreign materials, and colonies of microorganisms. The usual consequence of coronary embolism is acute myocardial infarction. Of over 200 hearts examined systematically at necropsy in patients with fatal coronary heart disease, one was observed to have an embolus of necrotic myocardium in a coronary artery. The patient, a 73-year-old woman, died suddenly 10 hours after onset of symptoms (chest pain) of acute myocardial infarction. A precordial murmur was never audible. Necropsy disclosed rupture of one left ventricular papillary muscle and a clump of myocardium in the lumen of the right coronary artery. The embolized myocardium was similar to that observed in the ruptured papillary muscle.

Myocardial embolus, to our knowledge, has not been reported previously in a coronary artery. The coronary embolus in the present patient probably would have been missed had not the entire major coronary tree been examined histologically, a method used to study the coronary arteries at necropsy in all of our over 200 fatal cases of coronary heart disease.

Publications: Hammer, W.J., Ferrans, V.J., and Roberts, W.C.: Myocardial Embolus to Coronary Artery. Result of Rupture of Papillary Muscle During Acute Myocardial Infarction. Chest 68:843-844, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03009-02 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Tuberous xanthoma in type II hyperlipoproteinemia.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bernadine H. Bulkley, Research Associate, Sect. Pathol., NHLI
OTHER: L. Maximilian Buja, Staff Fellow, Sect. Pathol., NHLI
Victor J. Ferrans, Vis. Scient., Sect. Pathol., NHLI
Gregory B. Bulkley, Clinical Associate, Surgery Branch NCI
William C. Roberts, Chief, Sect. Pathol., NHLI

COOPERATING UNITS (if any)

Surgery Branch, NCI

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.20	0.05	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

A description is presented of histologic, histochemical and ultrastructural features of lipid-containing foam cells in a tuberous xanthoma from a patient with homozygous type II hyperlipoproteinemia.

1095

PHS-6040
(12-75)

Project Description: Histologic, histochemical and ultrastructural studies of a tuberous xanthoma from a patient with homozygous type II hyperlipoproteinemia showed that virtually all of the lipid was within histiocytic foam cells; no lipid was identified in interstitial regions or in blood vessels. Primitive mesenchymal cells, elongated perivascular and fibroblast-like cells, and lysosome-filled macrophages also were present within the xanthoma, indicating possible stages in the evolution of dermal mesenchymal cells into mature, cholesterol-rich foam cells. Morphologically, the lipid was in 4 different forms: large droplets, which were the predominant form, and membrane-bound crystals, concentric lamellar bodies, and ceroid deposits.

Publications: Bulkley, B. H., Buja, L. M., Ferrans, V. J., Bulkley, G. B. and Roberts, W. C.: Tuberous xanthoma in homozygous type II hyperlipoproteinemia. A histologic, histochemical and electron microscopic study. Arch. Pathol. 99: 293-300, 1975

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03011-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Hypertensive Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. C. Roberts Chief, Sect. Pathol. NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI-NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report summarizes clinical frequencies of systemic hypertension and necropsy evidence of cardiomegaly in various cardiovascular conditions termed "the hypertensive diseases" because of their frequent association with systemic hypertension. Although long recognized as a major risk factor, systemic hypertension appears to be an even greater risk factor to development of various cardiovascular conditions than previously appreciated. Hypertension by itself appears to be the sole underlying factor in most cases of non-traumatic cerebral arterial or aortic rupture. In association with hyperlipidemia, hypertension clearly accelerates atherosclerosis and its devastating consequences.

1097

PHS-6040
(12-75)

Project Description: This report summarizes clinical frequencies of systemic hypertension and necropsy evidence of cardiomegaly in various cardiovascular conditions termed "the hypertensive diseases" because of their frequent association with systemic hypertension. Although long recognized as a major risk factor, systemic hypertension appears to be an even greater risk factor to development of various cardiovascular conditions than previously appreciated. Hypertension by itself appears to be the sole underlying factor in most cases of non-traumatic cerebral arterial or aortic rupture. In association with hyperlipidemia, hypertension clearly accelerates atherosclerosis and its devastating consequences.

This report demonstrates that systemic hypertension acts as a major risk factor to development of cardiovascular disease in 2 ways: 1) by increasing the deposition of atherosclerotic plaques (intimal lesions) in major arteries most commonly causing luminal narrowing with resulting organ ischemia or infarction or both, and 2) by weakening the media of certain arteries causing aneurysms which may or may not rupture. Its effect on the arterial media is directed whereas its effect on the arterial intima is indirect. Hypertension is the only known major underlying factor in 2 conditions: intracerebral microaneurysm (with or without rupture) and dissecting aortic aneurysm, both of which are associated with medial weakening or disruption. Reduction in blood pressure clearly leads to a reduction in the incidence of non-traumatic intracerebral hemorrhage and of dissecting aneurysm. The more common consequence of hypertension, however, is its ability to increase the amount of atherosclerotic plaquing in various arteries. This effect, however, is not direct because hypertension in population groups with normal serum cholesterol levels do not have more or larger atherosclerotic plaques than do the normotensive persons in those populations. Thus, hypertension accelerates or increases atherosclerosis only in hypercholesterolemic population groups and not in those with normal serum cholesterol levels.

Publications: Roberts, W.C.: The Hypertensive Diseases. Evidence that Systemic Hypertension is a Greater Risk Factor to the Development of Other Cardiovascular Diseases than Previously Suspected. Am. J. Med. 59:523-532, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03012-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cardiac Pathology After Valve Replacement by Disc Prosthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	M. C. Fishbein	Guest Worker, Sect. Pathol.	NHLI
	A. Golden	Chairman, Dept. Pathol., Georgetown Univ. Washington, D.C.	

COOPERATING UNITS (if any)

Department of Pathology, Georgetown University, Washington, D.C.

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ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and necropsy observations are described in 61 patients in whom one or more cardiac valves had been replaced with discoid prostheses (Hufnagel type).

1099

PHS-6040
(12-75)

Project Description: Clinical and necropsy observations are described in 61 patients who had one or more cardaic valves replaced with a discoid prosthesis of the Hufnagel type. The most common (31%) cause of death among the 45 patients who died early (less than 65 days after operation) appeared to be prosthetic disproportion; that is, the prosthesis was too big for the aorta or ventricular cavity into which it was inserted so that inadequate space was present between the margins of the disc and the endocardium of ventricle or intima of aorta. Prosthetic thrombosis occurred in only 3 of the 45 patients who died early, but poppet movement appeared considerably altered in each. In contrast, thrombi were observed on a prosthesis in 14 of the 16 patients who died late (4 to 47 months [avg 21] postoperatively), but in none did the thrombi appear of sufficient size to alter poppet function. Excessive bleeding occurred in 11 (24%) of the 45 early deaths and was primarily related to the insertion of a patch in the root of the aorta. Uncorrected valvular disease either by itself or by its ability to alter function of the prosthesis appeared responsible for death in 6 (13%) of the 45 patients who died early and in 2 (6%) of the 16 who died late. Insertion of a mitral poppet disc in a patient with uncorrected aortic regurgitation, even of mild degree, may be hazardous because the aortic regurgitant jet stream may interfere with proper function of the mitral disc. Likewise, insertion of a poppet disc only in the aortic valve position in a patient with combined aortic and mitral regurgitation may considerably increase the degree of mitral incompetence because the aortic prosthesis is intrinsically obstructive.

Disc wear or variance was observed in all but one prosthesis in place for more than 1 year. Although hemolytic anemia of significant degree was not observed in any of the 16 patients who died late, the occurrence of renal hemosiderosis in 13 of the 16 patients indicates that the poppet disc prosthesis is considerably traumatic to erythrocytes. Thus, this type of prosthesis is not an ideal substitute cardiac valve. It clots, despite anticoagulant therapy, it is intrinsically stenotic, portions of it, that is, the disc, degenerate, and it causes hemolysis to erythrocytes.

Publications: Roberts, W.C., Fishbein, M.C., and Golden, A.: Cardiac Pathology After Valve Replacement by Disc Prosthesis: A Study of 61 Necropsy Patients. Am. J. Cardiol. 35: 740-760, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03013-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Observations After Insertion of Hufnagel Prostheses in Descending Aorta

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	M. C. Fishbein	Guest Worker, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
Department of Pathology, Georgetown Univ., Washington, D.C.

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SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and necropsy observations are described in 2 patients who died 11 and 13 years, respectively, after implantation of Hufnagel prostheses (the first ever used successfully in humans to treat cardiac valve disease) in the descending thoracic aorta. Despite the long implantation periods, there was no evidence of prosthetic degeneration or thrombosis or intravascular hemolysis.

1101

PHS-6040
(12-75)

Project No. Z01 HL 03013-02 OD P

Project Description: Clinical and necropsy observations are described in two patients who died 11 and 13 years, respectively, after implantation of Hufnagel prostheses (the first ever used successfully in humans to treat cardiac valve disease) in descending thoracic aorta. Despite the long implantation periods, there was no evidence of prosthetic degeneration or thrombosis or intravascular hemolysis. Although approximately 4000 Hufnagel descending aortic prostheses were distributed by the manufacturer for human use, data in only 55 patients in whom these prostheses were inserted were found in previous publications, and 26 of them had died. Of the 13 late deaths previously reported, no evidence of prosthetic degeneration or thrombosis was described in the three patients surviving > 3 years (8, 10, 12.5 years, respectively). Since neither our patients nor any of the other three reported long-term survivors had prosthetic-related complications and since the dangers of excising the descending aortic prosthesis are considerable, it appears most reasonable, as a rule, not to remove the descending aortic prosthesis at any time in these patients if aortic valve replacement is subsequently performed.

Publications: Fishbein, M.C., and Roberts, W.C.: Late Postoperative Anatomic Observations after Insertion of Hufnagel Caged-ball Prostheses in Descending Thoracic Aorta. Chest 68:6-11, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03014-02 OD P

PERIOD COVERED
July 1, 1976 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Aortic Valve Atresia: A Necropsy Study of 73 Cases.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.C. Roberts	Chief, Sect. Pathol. NHLI
OTHER:	L.W. Perry	Div. Ped. Cardiology & Dept. Child Health & Dev. CHNMC, Washington, D.C.
	R.S. Chandra	Dept. Path. CHNMC Washington, D.C.
	S.R. Shapiro	Div. Ped. Cardiology CHNMC, Washington, D.C.
	L.P. Scott	Div. Ped. Cardiology CHNMC, Washington, D.C.
	G.E. Meyers	Fellow, Dept. Ped. & Path. George Washington University School Medicine Washington, D.C.

COOPERATING UNITS (if any)
Division of Pediatric Cardiology, Department of Child Health and Developmen and Department of Pathology, Children's Hospital National Medical Center, Washington, D.C.

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SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report describes the morphologic features of 73 hearts examined at necropsy with aortic valve atresia. The patients ranged in age from 1 to 17 days (average 5) and 74% were boys. Previous reports on aortic valve atresia described only the occurrence of a hypoplastic left ventricle with or without atretic mitral valves. In the present study of 73 cases 4 were found to have normal or near-normal sized left ventricles rather than hypoplastic ones and 3 of them had normally developed mitral valves.

1103

Project Description: Although a relatively uncommon lesion, aortic valve atresia is an important congenital cardiovascular malformation because it represents the least tolerated cardiac anomaly and, therefore, one extreme of the spectrum of congenital heart disease. Among infants with congenital malformations of the heart or great vessels during the first week of life, aortic valve atresia is the most common cause of death, the most common cause of congestive heart failure, and the cause of the largest hearts. This report describes the morphologic features of 73 hearts examined at necropsy with aortic valve atresia. The patients ranged in age from 1 to 17 days (average 5) and 74% were boys. Previous reports on aortic valve atresia described only the occurrence of a hypoplastic left ventricle with or without atretic mitral valves. In the present study of 73 cases 4 were found to have normal or near-normal sized left ventricles rather than hypoplastic ones and 3 of them had normally developed mitral valves. All 4 patients with normal or near-normal sized left ventricles had ventricular septal defects whereas all the remaining 69 with hypoplastic left ventricles had intact ventricular septae. Because no previous descriptions have appeared of ventricular septal defect or normal-sized left ventricles associated with aortic atresia a new classification of this condition was proposed. In essence, there are 2 types of aortic valve atresia: in type 1 the left ventricle is hypoplastic and the ventricular septum is intact. Among our 69 patients with this type, 25 had atretic mitral valves and 44 had hypoplastic mitral valves. In type II, the left ventricular cavity is of normal size or near-normal size and one or more ventricular septal defects are present. Among our 4 patients with this type, 1 had an atretic mitral valve and 3 had a normally developed mitral valve. Although operative intervention in patients with hypoplastic left ventricles associated with aortic atresia is fruitless, it appears that operative intervention may be fruitful in the individuals with normal or near-normal sized left ventricular cavities.

Publication: Roberts, W.C., Perry, L.W., Chandra, R.S., Shapiro, S.R., Lewis, P.S., Meyers, G.E.: Aortic Valve Atresia: A New Classification Based on Necropsy Study of 73 Cases. American Journal of Cardiology 37:753-757, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03015-03 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Endocardial Structure in Carcinoid Heart Disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Vis. Scient., Sect. Pathol., NHLI
OTHER: William C. Roberts, Chief, Sect. Pathol., NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.1	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

The endocardial lesions in carcinoid heart disease are characterized by the presence of smooth muscle cells, myofibroblasts and fibroblasts embedded in a stroma that is rich in collagen, microfibrils and basement membrane material, but lacks elastic fibers.

1105

PHS-6040
(12-75)

Project Description: The carcinoid syndrome is associated with pathognomonic cardiac lesions which consist of plaque-like fibrous thickenings in mural and valvular endocardium. Ultrastructural study of these plaques in right atrium (2 patients) and tricuspid and pulmonic valve (1 patient) showed similar features. Most cellular elements were mature smooth muscle cells which varied from fusiform to stellate in shape and had greatly thickened, reduplicated basement membranes. Extracellular components consisted of: layers of normal-appearing collagen fibrils oriented parallel to the surfaces of the plaques and arranged in a cross-weaving pattern; 100 to 200 A diameter microfibrils; layers of fibrillar material similar to basement membranes of smooth muscle cells, and dense spicules, 150 A diameter and up to 800 A in length. No elastic fibers or fibrin deposits were found. These findings suggest that carcinoid plaques result from stimulation of endocardial smooth muscle cells to produce collagen and basement membrane-like material; such stimulation may be intermittent, as evidenced by the layered arrangement of the plaques.

Publications: Ferrans, V.J., and Roberts, W.C.: Ultrastructure of endocardial plaques in carcinoid heart disease. Hum Pathol (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03016-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Unruptured Sinus of Valsalva Aneurysm

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHERS:	M. C. Fishbein	Guest Worker, Sect. Pathol.	NHLI
	Robert T. Obma	Internist, Skemp-Grandview Clinic, La Crosse, Wisconsin	

COOPERATING UNITS (if any)
Skemp-Grandview Clinic, La Crosse, Wisconsin

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

An unruptured congenital sinus of Valsalva aneurysm (behind the right aortic valve cusp) is described as an incidental necropsy finding in an 82-year-old man.

1107

PHS-6040
(12-75)

Project No. Z01 HL 03016-02 OD P

Project Description: An unruptured congenital sinus of Valsalva aneurysm (behind the right aortic valve cusp) is described as an incidental necropsy finding in an 82 year old man. Review of previous reports on aneurysms involving only one of the three aortic sinuses discloses that few cases have been described, and that these lesions are rarely diagnosed during life. It is probable, however, that unruptured aortic sinus aneurysm (involving only one sinus) is more common than previous reports indicate, but that, among patients with congenital sinus aneurysm, rupture is likely to occur.

Publications: Fishbein, M.C., Obma, R., and Roberts, W.C.: Unruptured Sinus of Valsalva Aneurysm. Am. J. Cardiol. 35: 918-922, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03017-02 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Ultrastructure of endocardial papillary elastofibromas.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael C. Fishbein	Guest Worker	Sect. Pathol. , NHLI
OTHER:	Victor J. Ferrans	Vis. Scient.	Sect. Pathol. , NHLI
	William C. Roberts	Chief	Sect. Pathol. , NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.30	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

Ultrastructural studies of endocardial papillary elastofibromas disclosed that each tumor papilla is composed of a dense, central core of collagenous and elastic fibers; a peripheral myxomatous zone with deposits of acid mucopolysaccharides, and an overlying layer of endothelial cells.

1109

PHS-6040
(12-75)

Project No. Z01 HL 03017-02 OD P

Project Description: The organization of cellular and extracellular components appeared similar and was distinctive in 3 endocardial papillary elastofibromas studied by light and electron microscopy. Each tumor papilla contained: 1) a dense, central core of collagen and elastic tissue; 2) a peripheral, myxomatous layer with deposits of acid mucopolysaccharides, and 3) an overlying, hyperplastic layer of endothelial cells. The cells in all 3 zones had numerous cytoplasmic filaments, 100 A in diameter, and dilated cisterns of endoplasmic reticulum; endothelial cells also had intercellular junctions and numerous pinocytotic vesicles. The myxomatous stroma varied from amorphous to fibrillar, and the collagenous cores showed focal degeneration.

Publications: Fishbein, M.C., Ferrans, V. J. and Roberts, W. C.: Endocardial papillary elastofibromas. Arch. Pathol. 99: 335-341, 1975

1110

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03018-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Ultrastructural features of endocardial fibroelastosis.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael C. Fishbein	Guest Worker	Sect. Pathol., NHLI
OTHER:	Victor J. Ferrans	Vis. Scient.	Sect. Pathol., NHLI
	William C. Roberts	Chief	Sect. Pathol., NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
ODIR

SECTION
Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

Histological and ultrastructural studies revealed that the average size of elastic fibers in thickened endocardium was much larger in congenital than in acquired endocardial fibroelastosis.

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PHS-6040
(12-75)

Project Description: Histological and ultrastructural studies of left ventricular endocardium from 10 patients with endocardial fibroelastosis (EFE) revealed that the average size of the elastic fibers in thickened endocardium was much larger in the 4 patients with congenital EFE than in the 6 patients with acquired EFE (secondary to ischemic heart disease in 2 patients, to prosthetic cardiac valves in 3, and to irradiation to the chest in 1). Both components of normal elastic tissue (central, amorphous cores and peripheral microfibrils) were present in endocardial elastic fibers from each patient. Ultrastructural identification of elastic fibers was facilitated by staining with silver tetraphenylporphin sulfonate.

Publications: Fishbein, M.C., Ferrans, V. J. and Roberts, W. C.: Histologic and ultrastructural features of primary and secondary fibroelastosis. Arch. Pathol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03019-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Morphologic evaluation of myocardial protection.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans Vis. Scient. Sect. Pathol., NHLI
OTHER: None.

COOPERATING UNITS (if any)
None.

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.05	0.05

SUMMARY OF WORK (200 words or less - underline keywords)

This project was designed to review histologic, histochemical and electron microscopic methods that are useful in the morphologic evalation of cardiac tissues subjected to procedures designed to prevent or reduce myocardial ischemic or metabolic injury during cardiac operations.

1113

PHS-6040
(12-75)

Project No. Z01 HL 03019-02 OD P

Project Description: This presentation reviews histologic, histochemical and electron microscopic methods considered useful in the morphologic evaluation of procedures designed to prevent myocardial ischemic or metabolic injury during cardiac operations. Conclusions of this review are:

1. Transmural samples of myocardium should be studied as the response of the ventricular walls to ischemic injury is not homogeneous.
2. Collection of samples should be continued until the injury reaches a stable end point.
3. Studies of myocardial protection should take into account the fact that ischemic injury is modified considerably by reflow phenomena.
4. Ultrastructural studies are indispensable, and histologic methods are of limited value in the morphologic evaluation of early myocardial injury.

Publications: Ferrans, V.J.: Morphological methods for evaluation of myocardial protection. Ann Thorac Surg 20: 11-20, 1975

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03021-02 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Massive Myocardial Hemosiderosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. N. Arnett	Staff Associate, Sect. Pathol.	NHLI
OTHER:	A. W. Nienhuis	Head. Sect. Clin. Hematology	NHLI
	W. L. Henry	Senior Investigator, Cardiol. Br.	NHLI
	V. J. Ferrans	Senior Investigator, Sect. Pathol.	NHLI
	D. R. Redwood	Head, Sect. Cardiovasc. Diagnosis	NHLI
	W. C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

Molecular Hematology and Cardiology Branches, National Heart & Lung Institute, Bethesda, Maryland

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report discusses a 23-year-old man with Blackfan-Diamond anemia. He received over 500 transfusions during his life and the effects of the massive deposition of iron in the myocardium is reviewed.

1115

PHS-6040
(12-75)

Project No. Z01 HL 03021-02 OD P

Project Description: This report discusses a 23-year-old man with Blackfan-Diamond anemia. He received over 500 transfusions during his life and the effects of the massive deposition of iron in the myocardium is reviewed. This report emphasizes that heavy deposition of iron in myocardial fibers causes the myocardium to function abnormally and the present patient developed evidence of congestive failure and various arrhythmias. The distribution of iron in myocardium was described and the amount was quantitated biochemically and also studied ultrastructurally.

Publications: Arnett, E.N., Nienhuis, A.W., Henry, W.L., Ferrans, V.J., Redwood, D.R., and Roberts, W.C.: Massive Myocardial Hemosiderosis: A Structure-Function Conference at the National Heart and Lung Institute. Am. Heart J. 90: 777-787, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03023-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cardiac structure in hypertrophy.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Vis. Scient. , Section of Pathology, NHLI
OTHER: None.

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0

SUMMARY OF WORK (200 words or less - underline keywords)

A detailed review of qualitative and quantitative ultrastructural changes in cardiac hypertrophy.

1117

PHS-6040
(12-75)

Project Description: This project consisted of a detailed review of cardiac structure in hypertrophy, with emphasis on ultrastructural alterations of myocardium in the three stages of hypertrophy (developing hypertrophy, stable hyperfunction and cellular exhaustion) and on quantitative data derived from stereological analysis of electron micrographs from animal models of hypertrophy.

Publications: Ferrans, V.J.: Cardiac structure in hypertrophy. To be published as a book chapter in Cardiac Hypertrophy, Morkin, E. (Ed.), John Wiley & Sons

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03025-03 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Intranuclear glycogen in myocardium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Vis. Scient.	Sect. Pathol., NHLI
OTHER:	Barry J. Maron	Sen. Invest.	CB NHLI
	L. Maximilian Buja	Staff Fellow	Sect. Pathol., NHLI
	Nayab Ali	Chief, Cardiac Laboratory,	D.C. General Hospital
	William C. Roberts	Chief	Sect. Pathol., NHLI

COOPERATING UNITS (if any)

Cardiology Branch, NHLI
Cardiac Laboratory, District of Columbia General Hospital, Washington, DC

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SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.40	0.25	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

Glycogen deposits were observed within nuclei of cardiac muscle cells in 6 of 90 myocardial biopsies from patients with various types of heart disease. The ultrastructure and cytochemistry of these glycogen deposits was described and a detailed review of the literature on intranuclear glycogen was presented.

1119

PHS-6040
(12-75)

Project No. Z01 HL 03025-03 OD P

Project Description: Ultrastructural and cytochemical studies of myocardial biopsies disclosed the presence of glycogen deposits within nuclei of hypertrophied and normal-sized cardiac muscle cells in 6 (7%) of 90 patients with various cardiac diseases. Intranuclear glycogen appeared as β-particles, 160 to 360 Å in diameter, which either formed small aggregates or were dispersed in the nucleoplasm. Cells containing intranuclear glycogen did not show damage or degeneration. It is concluded that under certain conditions the nuclei of cardiac muscle cells can acquire the capacity to synthesize glycogen.

Publications: Ferrans, V. J., Maron, B. J., Buja, L. M., Ali, N. and Roberts, W. C.: Intranuclear glycogen deposits in human cardiac muscle cells: ultrastructure and cytochemistry. J. Molec. Cell. Cardiol. 7: 697-702, 1975.

1120

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03026-03 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cardiac lesions in bone marrow transplantation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: L. Maximilian Buja Staff Fellow, Sect. Pathol. NHLI
OTHER: Victor J. Ferrans Vis. Scient., Sect. Pathol. NHLI
Robert G. Graw Chief, Exp. Hematol. Section, PO, NCI

COOPERATING UNITS (if any)
Experimental Hematology Section, Pediatric Oncology Branch, NCI

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SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This study describes cardiac pathologic findings in patients who received bone marrow transplantation for therapy of anemias, immune deficiency diseases or neoplasms. Some of the changes observed were related to complications of the underlying disease processes; others were side effects of the chemotherapeutic agents used in the treatment of neoplasms or in the process of immunosuppression, and others probably represented manifestations of abnormal immune mechanisms and of graft-versus-host disease.

1121

Project Description: Cardiac pathologic findings were analyzed in 20 necropsied patients from a series of 26 patients with leukemia, aplastic anemia, immune deficiency disease or metastatic cancer who had been treated with bone marrow transplantation. Most cardiac alterations were similar to those which occur in patients with hematologic and neoplastic diseases who have not been treated with bone marrow transplantation. Other cardiac alterations were more specifically related to bone marrow transplantation. Six patients exhibited a distinctive interstitial reactive change characterized by the presence of a pleomorphic population of lymphoid, histiocytic and Anitschkow cells. This alteration may have been induced by abnormal immune mechanisms. Two patients developed fatal cardiac failure in the post-transplant period, and exhibited myocardial damage with histologic and ultrastructural features indicative of severe acute injury. Clinicopathologic analysis strongly suggested that the fatal cardiotoxicity in both patients resulted primarily from effects of high doses of cyclophosphamide (180 mg/kg and 270 mg/kg) which were administered as part of a newly developed regimen of combination chemotherapy-immunosuppression (B.A.C.T.).

Publications: Buja, L.M., Ferrans, V.J. and Graw, R. G.: Cardiac pathologic findings in patients treated with bone marrow transplantation. Hum Pathol 7: 17-45, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03027-02 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
The Structural Basis of Abnormal Cardiac Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. C. Roberts Chief, Sect. Path. NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report, in essence, is a review of morphologic observations made in this laboratory during the past 10 years regarding coronary, hypertensive, valvular, idiopathic myocardial and pericardial heart disease.

1123

PHS-6040
(12-75)

Project Description: This report, in essence, is a review of morphologic observations made in this laboratory during the past 10 years regarding coronary, hypertensive, valvular, idiopathic myocardial and pericardial heart diseases. The status of the major extramural coronary arteries in patients with fatal ischemic heart disease is summarized. Specifically, for fatal or even symptomatic ischemic heart disease to occur, at least 2 of the 3 major coronary arteries must be >75% narrowed by old atherosclerotic plaques. The myocardial response to severe coronary narrowing is highly variable and indeed no differences in degrees of luminal narrowing were observed among fatal cases of angina pectoris, sudden coronary death, and acute myocardial infarction. The frequency of histories of systemic hypertension in many cardiovascular conditions was reviewed and secondly the percent of patients with various cardiovascular conditions who have cardiomegaly were reviewed. It was emphasized that systemic hypertension is a far greater risk factor than previously emphasized by history of hypertension or measurement of elevated blood pressure if left ventricular hypertrophy is used as an indicator of hypertension rather than history or actual measurement of blood pressure. The morphologic features of various valvular lesions were reviewed and emphasis was placed on the fact that rheumatic heart disease is a less common cause of valvular heart disease than are non-rheumatic etiologies. Stress was placed on classifying valvular heart disease into the purely regurgitant lesions and into those with elements of stenosis because the etiology of the former are only 3 in number whereas the etiology of the latter were numerous. Morphologic features of the idiopathic cardiomyopathies, i.e., both the ventricular dilated and the non-ventricular dilated type (ASH), were reviewed. Likewise, various morphologic features of pericardial heart disease were reviewed.

Publications: Roberts, W.C.: The Structural Basis of Abnormal Cardiac Function: A Look at Coronary, Hypertensive, Valvular, Idiopathic Myocardial, and Pericardial Heart Diseases. In Levine, H.J. (Ed.): Clinical Cardiovascular Physiology. New York, Grune & Stratton, 1976, pp. 1-56.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03028-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Prosthetic Valve Endocarditis Due To Listeria Monocytogenes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.H. Breyer	Surgical Associate, Surgical Branch	NHLI
OTHER:	E.N. Arnett	Staff Associate, Sect. Pathol.	NHLI
	W.C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

Surgical Branch NHLI

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report summarized clinical and morphologic observations in a patient with prosthetic valve endocarditis due to Listeria Monocytogenes. There are no previous reports of prosthetic valve endocarditis due to this particular organism.

1125

PHS-6040
(12-75)

Project No. Z01 HL 03028-01 OD P

Project Description: This report summarizes clinical and morphologic observations in a patient with prosthetic valve endocarditis due to Listeria Monocytogenes. There are no previous reports of prosthetic valve endocarditis due to this particular organism.

Publications: Breyer, R.H., Arnett, E.N., Roberts, W.C.: Prosthetic Valve Endocarditis Due to Listeria Monocytogenes. Journal of Thoracic and Cardiovascular Surgery (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03029-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Prosthetic Valve Endocarditis: A Clinicopathologic Study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.N. Arnett	Staff Associate, Sect. Pathol.	NHLI
OTHER:	W.C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
None

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SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and morphologic features are described in 23 necropsy patients with endocarditis involving rigid-framed prosthetic valves: aortic in 16 patients, and mitral in 7. Comparison of observations in the 23 patients with prosthetic valve endocarditis to those in 74 patients with active infective endocarditis involving natural left-sided cardiac valves showed significant ($p < 0.05$) differences in percent with ring abscess, hemodynamic consequences of the endocarditis (valve stenosis), frequency of staphylococcus as the causative organism, and percent with complete heart block or left bundle branch block.

1127

PHS-6040
(12-75)

Project Description: Clinical and morphologic features are described in 23 necropsy patients with endocarditis involving rigid-framed prosthetic valves: aortic in 16 patients, and mitral in 7. The interval from valve replacement to onset of symptoms of prosthetic valve endocarditis was less than 2 months in 9 patients and longer than 2 months in 14 patients. The most frequent infecting organism was the staphylococcus (14 patients). In each of the 23 patients the infection was located behind the site of attachment of the prosthesis to the valve ring, and the infection spread to adjacent structures in 14 patients, 12 of whom had aortic prostheses. Prosthetic detachment causing severe regurgitation occurred in 12 of the 15 patients with infected aortic prostheses, and in 2 of the 7 patients with infected mitral prostheses. Prosthetic obstruction by vegetative material occurred in 5 of 7 patients with prosthetic mitral infection and in only 2 of 15 with prosthetic aortic infection. Seven patients with aortic prosthetic valve endocarditis developed high degrees of conduction defects: complete heart block in 5, and complete left bundle branch block in 2.

Comparison of observations in the 23 patients with prosthetic valve endocarditis to those in 74 patients with active infective endocarditis involving natural left-sided cardiac valves showed significant ($p \leq 0.05$) differences in percent with ring abscess, hemodynamic consequences of the endocarditis (valve stenosis), frequency of staphylococcus as the causative organism, and percent with complete heart block or left bundle branch block. No significant differences were observed between the 2 groups when comparing age, sex, type of underlying valve disease, or frequency of organ infarcts or splenomegaly.

Publications: Arnett, E.N., Roberts, W.C.: Prosthetic Valve Endocarditis: A Clinicopathologic Study. Book entitled PROSTHETIC VALVE ENDOCARDITIS published by University Park Press (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03030-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Angiographically Normal Coronary Arteries After Healing of Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.N. Arnett	Staff Associate, Sect. Pathol.	NHLI
OTHER:	W.C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report examines previous reports of myocardial infarction and angiographically normal coronary arteries and discusses possible explanations for the occurrence of myocardial infarction in normal coronary arteriograms. Five explanations are considered and evidences presented that the acute myocardial infarction was caused by an occluding embolus which subsequently lysed or recanalized.

1129

PHS-6040
(12-75)

Project Description: This report examines previous reports of myocardial infarction and angiographically normal coronary arteries and discusses possible explanations for the occurrence of myocardial infarction in normal coronary arteriograms. There have been 45 reported patients with myocardial infarction and angiographically normal coronary arteries: in 5 the acute myocardial infarction was produced at the time of cardiac catheterization and in the other 40 patients acute myocardial infarction was unrelated to cardiac catheterization. In 39 of the latter 40 patients with non-catheter related infarctions, the interval between the onset of acute myocardial infarction and performance of coronary angiography was longer than 2 months. Thus, the status of the coronary arteries at the time when a portion of left ventricular myocardium was necrotic is uncertain. Indeed, a normal coronary arterial tree has never been demonstrated by angiography at the time of acute myocardial infarction. This report examines, then, how a coronary tree may be entirely normal by angiography after healing of an acute myocardial infarction. Five explanations are considered and evidences presented that the acute myocardial infarction was caused by an occluding embolus which subsequently lysed or recanalized.

Publications: Arnett, E.N., Roberts, W.C.: Angiographically Normal Coronary Arteries After Healing of Acute Myocardial Infarction. Practical Cardiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03031-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Prosthetic Valve Endocarditis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.N. Arnett	Staff Associate, Sect. Pathol.	NHLI
OTHER:	W.C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and morphologic features are described in 22 necropsy patients with endocarditis involving rigid-framed prosthetic valves: aortic in 15 patients, and mitral in 7. Comparison of observations in the 22 patients with prosthetic valve endocarditis to those in 74 patients with active infective endocarditis involving natural left-sided cardiac valves showed significant ($p < 0.05$) differences in percent with ring abscess, hemodynamic consequences of the endocarditis (valve stenosis), frequency of Staphylococcus as the causative organism, and percent with complete heart block or left bundle branch block.

1131

PHS-6040
(12-75)

Project No. Z01 HL 03031-01 OD P

Project Description: Clinical and morphologic features are described in 22 necropsy patients with endocarditis involving rigid-framed prosthetic valves: aortic in 15 patients, and mitral in 7. The interval from valve replacement to onset of symptoms of prosthetic valve endocarditis was less than 2 months in 8 patients and longer than 2 months in 14 patients. The most frequent infecting organism was the Staphylococcus (13 patients). In each of the 22 patients the infection was located behind the site of attachment of the prosthesis to the valve ring, and the infection spread to adjacent structures in 13 patients, 11 of whom had aortic prostheses. Prosthetic detachment causing severe regurgitation occurred in 12 of the 15 patients with infected aortic prostheses, and in 2 of the 7 patients with infected mitral prostheses. Prosthetic obstruction by vegetative material occurred in 5 of 7 patients with prosthetic mitral infection and in only 1 of 15 with prosthetic aortic infection. Seven patients with aortic prosthetic valve endocarditis developed high degrees of conduction defects: complete heart block in 5, and complete left bundle branch block in 2.

Comparison of observations in the 22 patients with prosthetic valve endocarditis to those in 74 patients with active infective endocarditis involving natural left-sided cardiac valves showed significant ($p < 0.05$) differences in percent with ring abscess, hemodynamic consequences of the endocarditis (valve stenosis), frequency of Staphylococcus as the causative organism, and percent with complete heart block or left bundle branch block. No significant differences were observed between the 2 groups when comparing age, sex, type of underlying valve disease, or frequency of organ infarcts or splenomegaly.

Publications: Arnett, E.N., Roberts, W.C.: Prosthetic Valve Endocarditis: A Clinicopathologic Analysis of 22 Necropsy Patients with Comparison of Observations to 74 Necropsy Patients with Active Infective Endocarditis Involving Natural Left-Sided Cardiac Valves. American Journal of Cardiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03032-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pericardial Heart Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	T.L. Spray	Staff Associate Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175hrs.	175hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report reviews morphologic aspects of pericardial heart disease. A morphologic classification for this condition is presented.

1133

PHS-6040
(12-75)

Project No. ZO1 HL 03032-01 OD P

Project Description: This report reviews morphologic aspects of pericardial heart disease. A morphologic classification for this condition is presented. An ideal classification of pericardial heart disease obviously would take into account clinical, etiologic and morphologic features of this condition but a single classification combining these 3 components is lacking. Pericardial heart disease is relatively uncommon clinically and when present at necropsy it usually had not been recognized during life. The term "pericarditis" is inaccurate because most pericardial diseases are non-inflammatory in nature. Morphologically chronic pericardial heart disease may present clinically as an acute illness. Even when clinical symptoms are present, however, few patients develop evidence of cardiac dysfunction (constriction). When pericardial "constriction" occurs, it is the result of increased pericardial fluid or increased pericardial tissue or both. Increased fluid is treated by drainage; increased tissue is treated by excision. In most patients with chronic constrictive pericarditis the etiology is not apparent even after histologic examination of the patient.

Publications: Roberts, W.C., Spray, T.L.: Pericardial Heart Disease: A Study of its Causes, Consequences and Morphologic Features. Cardiovascular Clinics (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03033-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cocking of a poppet-disc prosthesis in the aortic position.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.J. Hammer	Fellow in Cardiology Washington, D.C.	Georgetown University
OTHER:	M.J. Hearne	Fellow in Cardiology Washington, D.C.	Georgetown University
	W.C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

Department of Medicine (Cardiology) and Pathology, Georgetown University Medical Center, Washington, D.C.

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Intermittent aortic regurgitation due to cocking is described for the first time after replacement of the aortic valve with a poppet-disc prosthesis. A combination of disc grooving and strut thrombus produced the cocking with resultant aortic regurgitation.

1135

PHS-6040
(12-75)

Project No. Z01 HL 03033-01 OD P

Project Description: Among patients with prosthetic cardiac valves, a common cause of both early and late death is prosthetic dysfunction. Auscultation and phonocardiographic abnormalities in these patients often are clues to such dysfunction. Peribasilar communications or improper seating of the poppet as a result of thrombus or variance are the usual causes of regurgitant type murmurs. This report was prompted by observing intermittent aortic regurgitation after aortic valve replacement with a poppet disc prosthesis. The mechanism of the regurgitation has not been recorded previously after aortic valve replacement.

Publications: Hammer, W.J., Hearne, M.J., Roberts, W.C.: Cocking of a Poppet-disc Prosthesis in the Aortic Position. A cause of intermittent aortic regurgitation. Journal of Thoracic and Cardiovascular Surgery 71: 259-261, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03034-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Prolapse of the Mitral Valve (Floppy Valve)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	D.L. Glancy	Dept. Med. Hotel Dieu Hospital	New Orleans La.
	R.P. Seningen	Dept. Diagn. Radiology	Clinical Center NIH
	B.J. Maron	Card. Branch	NHLI
	S.E. Epstein	Chief, Cardiol. Branch	NHLI

COOPERATING UNITS (if any)

Department of Radiology, Clinical Center, NIH and Cardiology Branch, NHLI.

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Prolapse of the mitral valve is described in 2 patients with the Ebstein's anomaly of the tricuspid valve. This association has not been described previously. It is probable, however, that this association is not a rare one, but that clinical features of the prolapsing mitral valve are obscured by those resulting from the malformed tricuspid valve.

1137

PHS-6040
(12-75)

Project Description: Prolapse of the mitral valve is described in 2 patients with the Ebstein's anomaly of the tricuspid valve. This association has not been described previously. It is probable, however, that this association is not a rare one, but that clinical features of the prolapsing mitral valve are obscured by those resulting from the malformed tricuspid valve. Opportunity also was provided to study anatomically the mitral valve of a patient known to have a systolic click and a late systolic murmur (Barlow's syndrome). Although there have been several anatomic descriptions of floppy mitral valves at necropsy, they have been extremely rare in patients known to have classic auscultatory features of the Barlow syndrome.

Publications: Roberts, W.C., Glancy, D.L., Seningen, R.P., Maron, B.J., Epstein, S.E.: Prolapse of the Mitral Valve (Floppy Valve) Associated with Ebstein's Anomaly of the Tricuspid Valve. American Journal of Cardiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 03035-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Combined Acute Rheumatic Fever and Congenitally Bicuspid Aortic Valve

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.C. Roberts	Chief, Sect. Pathol. NHLI
OTHER:	R.C. McReynolds	Staff Associate Sect. Pathol. NHLI
	Nayab Ali	Cardiologist D.C. General Hospital Wash. D.C.
	Michael Cuadra	Chief, Autopsy Service, D.C. General Hospital Washington, D.C.

COOPERATING UNITS (if any)
Departments of Medicine and Pathology
D.C. General Hospital, Washington, D.C.

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs	0

SUMMARY OF WORK (200 words or less - underline keywords)

An 18-year-old man is described in whom rheumatic heart disease, as evidenced by the presence of classic Aschoff bodies, occurred in combination with a congenitally bicuspid (purely incompetent) aortic valve. To our knowledge, this is the first report documenting the presence of rheumatic heart disease and congenitally bicuspid aortic valve in the same patient.

1139

PHS-6040
(12-75)

Project No. Z01 HL 03035-01 OD P

Project Description: An 18-year-old man is described in whom rheumatic heart disease, as evidenced by the presence of classic Aschoff bodies, occurred in combination with a congenitally bicuspid (purely incompetent) aortic valve. To our knowledge, this is the first report documenting the presence of rheumatic heart disease and congenitally bicuspid aortic valve in the same patient.

Publications: McReynolds, R.A., Ali, N., Cuadra, M., Roberts, W.C.: Combined Acute Rheumatic Fever and Congenitally Bicuspid Aortic Valve: A Hitherto Unconfirmed Combination. Chest 70: 98-100, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03036-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cardiac Pathology After Valve Replacement With A Tilting Disc Prosthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	W. J. Hammer	Fellow in Cardiology,	Georgetown University Washington, D.C.

COOPERATING UNITS (if any)

Georgetown University Washington, D.C.

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and necropsy observations are described in 46 patients who had one or more cardiac valves replaced with a tilting disc prosthesis of the Björk-Shiley type and these observations are compared to those made earlier in patients with caged-ball, caged-disc and trileaflet prostheses.

Although it is prone to clot and therefore requires long term anti-coagulation, the tilting disc (Björk-Shiley) prosthesis is not intrinsically stenotic or significantly incompetent, it is only minimally traumatic to erythrocytes, and, at this point in time, there is no evidence of prosthetic degeneration or variance.

1141

PHS-6040
(12-75)

Project Description: Clinical and necropsy observations are described in 46 patients who had one or more cardiac valves replaced with a tilting-disc prosthesis of the Björk-Shiley type and these observations are compared to those made earlier in patients with caged-ball, caged-disc and tri-leaflet prostheses. In comparison to its frequent occurrence in patients studied earlier with peripheral flow prostheses, either caged ball or caged disc, prosthetic dysfunction from prosthetic disproportion (too small a ventricular cavity or aortic root to freely accomodate the prosthesis) was observed in only two of the 46 patients with the semi-central flow Björk-Shiley prosthesis. Significant trauma to erythocytes, as determined by the presence of renal hemosiderosis, occurred in only 1 of 11 deaths after Björk-Shiley valve replacement; it occurred more frequently and to a far greater severity in the patients dying late after valve replacement with either caged-ball or caged-disc prostheses. Prosthetic thrombi may be less frequent after valve replacement with a tilting disc than after replacement with either a caged ball or caged disc. They occurred in 7 of 13 late deaths after Björk-Shiley replacement, but 4 of the 7 had infective prosthetic endocarditis, and of the remaining 4, only one had been on Warfarin sodium. In contrast, prosthetic thrombi were observed in 81 and 88 per cent, respectively, of 112 patients dying late after replacements with either caged-ball or caged-disc prostheses, and most of the 112 patients had received anticoagulants. Although the implantation periods were relatively short (3 to 30 months [avg 11]), prosthetic wear or variance of the tilting disc prosthesis did not occur in any of the 13 patients dying late.

Although it is prone to clot and therefore requires long term anti-coagulation, the tilting disc (Björk-Shiley) prosthesis is not intrinsically stenotic or significantly incomptent, it is only minimally traumatic to erythrocytes, and, at this point in time, there is no evidence of prosthetic degeneration or variance.

Publications: Roberts, W.C., Hammer, W.J.: Cardiac Pathology After Valve Replacement With A Tilting Disc Prosthesis (Björk-Shiley Type): A Study of 46 Necropsy Patients and 49 Björk-Shiley Prostheses. American Journal of Cardiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03037-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Choosing A Substitute Cardiac Valve: Type, Size, Surgeon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W.C. Roberts Chief, Sect. Pathol. NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, BETHESDA, MARYLAND 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This piece summarizes some ideal characteristics of substitute cardiac valves. It analyzes them in terms of the four basic types of presently available substitute cardiac valves. Of the four, the caged-disc prosthesis is the least desirable. Ideally the caged-ball prosthesis might be limited to those patients with volume lesions, i.e., pure or at least predominant regurgitation, because of the problem of prosthetic disproportion. The tilting-disc has favorable hemodynamic and wear characteristics but long-term anticoagulatants are essential as with the caged ball and caged disc type of prosthesis. The porcine xenograft at this time, appears quite favorable but sufficient (> 5 years) implantation periods for proper evaluation are just beginning to appear, and, therefore, final judgment must be reserved.

1143

Project Description: This piece summarizes some ideal characteristics of substitute cardiac valves. It analyzes them in terms of the four basic types of presently available substitute cardiac valves. Of the four, the caged-disc prosthesis is the least desirable: it obstructs, it clots, it degenerates, in each of the other three types the substitute cardiac valves have more favorable characteristics. Ideally the caged-ball prosthesis might be limited to those patients with volume lesions, i.e., pure or at least predominant regurgitation, because of the problem of prosthetic disproportion. The tilting-disc has favorable hemodynamic and wear characteristics but long-term anticoagulants are essential as with the caged ball and caged disc type of prosthesis. The porcine xenograft at this time, appears quite favorable but sufficient (> 5 years) implantation periods for proper evaluation are just beginning to appear, and, therefore, final judgment must be reserved. Assuming that valve replacement is to be carried out in a large medical center by a surgical team experienced in this procedure, the results of this operation today are most dependent on the type of, and size of, substitute valve inserted rather than on the surgeon who inserts the valve. Therefore, it is important for the cardiologist to become more informed on the various types of prosthetic cardiac valves and to become a member of the team deciding which type and size of cardiac valve is most proper for his (or her) patient.

Publications: Roberts, W.C.: Choosing a Substitute Cardiac Valve: Type, Size, Surgeon. American Journal of Cardiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HO 03038-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Tension On Coronary Bypass Conduits

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.L. Spray	Staff Associate	Sect. Pathol.	NHLI
OTHER:	W.C. Roberts	Chief, Sect. Pathol.		NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Of 41 saphenous vein aortocoronary bypass grafts from 21 patients, abnormal graft tension was judged to be present at necropsy in 6 grafts (15%) from 5 patients (24%). The abnormal tension appeared to result from the insertion of grafts too short to accomodate normal or abnormal distention of the right atrium and ventricle (graft to distal right coronary or posterior descending coronary artery) or of the pulmonary trunk (graft to left coronary system) after discontinuation of cardiopulmoanry bypass or development of acute congestive cardiac failure.

1145

PHS-6040
(12-75)

Project Description: Of 41 saphenous vein aortocoronary bypass grafts from 21 patients, abnormal graft tension was judged to be present at necropsy in 6 grafts (15%) from 5 patients (24%). Five (33%0 of 15 grafts placed to the right coronary system and one (3%) of 26 grafts to the left system were under excess tension. The abnormal tension appeared to result from the insertion of grafts too short to accomodate normal or abnormal distention of the right atrium and ventricle (graft to distal right coronary or posterior descending coronary artery) or of the pulmonary trunk (graft to left coronary system) after discontinuation of cardiopulmonary bypass or development of acute congestive cardiac failure.

Publications: Spray, T.L., Roberts, W.C.: Tension On Coronary Bypass Conduits: A Neglected Cause of Real or Potential Obstruction of Saphenous Vein Grafts. Journal of Thoracic and Cardiovascular Surgery (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03039-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Pure Angina Pectoris: Morphologic Features

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	T.L. Spray	Staff Associate, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Certain clinical and morphologic observations are described in 27 patients with severe pure angina pectoris. During life none had had clinical evidence of acute myocardial infarction or congestive cardiac failure. At necropsy, each had diffuse, extensive coronary atherosclerosis with severe luminal narrowing. Despite the severe coronary narrowing, there was relatively little myocardial damage.

1147

PHS-6040
(12-75)

Project Description: Certain clinical and morphologic observations are described in 27 patients with severe pure angina pectoris: 24 died during or shortly after cardiac operations designed to increase myocardial oxygenation and three died during cardiac catheterization. During life none had had clinical evidence of acute myocardial infarction or congestive cardiac failure. At necropsy, each had diffuse, extensive coronary atherosclerosis with severe luminal narrowing: the lumens of at least two, and average of 3.0, of the four major epicardial coronary arteries were narrowed greater than 75% in cross-sectional area by old atherosclerotic plaques. In a third of the patients, the lumens of each of the four major coronary arteries were narrowed to this extent. Despite the severe coronary narrowing, there was relatively little myocardial damage. Left ventricular scarring, all of relatively mild extent, was observed grossly in 52% of the 27 patients, but in each it involved only a small portion of myocardial wall. The left ventricular cavity was of normal weight in 56% of the 27 patients, and the average increase above the upper range of normal for all 27 patients was only 5%.

Thus, pure, clinically severe, angina pectoris is associated with severe diffuse coronary luminal narrowing but relatively little myocardial damage.

Publications: This piece will appear in a chapter of a book entitled ANGINA PECTORIS edited by Michael Oliver, M.D. to be published by Butterworth & Company in late 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03040-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Coronary Arteries and Left Ventricle in Clinically Isolated Angina Pectoris

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts Chief, Sect. Pathol. NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Certain clinical and morpholgoic observations are described in 27 patients with severe isolated angina pectoris of either the stable (five patients) or the unstable form (22 patients). During life none had had clinical evidence of acute myocardial infarction or congestive cardiac failure. At necropsy, each had diffuse, extensive coronary atherosclerosis with severe luminal narrowing. Despite the severe coronary narrowing, there was little myocardial damage.

1149

Project Description: Certain clinical and morphologic observations are described in 27 patients with severe isolated angina pectoris of either the stable (five patients) or the unstable form (22 patients). Twenty-four patients died during or shortly after cardiac operations designed to increase myocardial oxygenation and three died during cardiac catheterization. During life none had had clinical evidence of acute myocardial infarction or congestive cardiac failure. At necropsy, each had diffuse, extensive coronary atherosclerosis with severe luminal narrowing: the lumens of at least two, an average of 3.0, of the four major epicardial coronary arteries were narrowed > 75% in cross-sectional area by old atherosclerotic plaques. Despite the severe coronary narrowing, there was little myocardial damage. Left ventricular scarring (excluding papillary muscle) was observed grossly in only 14 (52%) of the 27 patients and in each it involved only a small portion of myocardial wall. The left ventricular cavity was of normal size in all except two patients. The hearts were of normal weight in 15 (56%) patients, and the average increase above the upper range of normal for all 27 patients was only 5%.

Thus, clinically isolated, severe angina pectoris is associated with severe diffuse luminal narrowing but relatively little myocardial damage.

Publications: Roberts, W.C.: The Coronary Arteries and Left Ventricle In Clinically Isolated Angina Pectoris, A Necropsy Analysis. Circulation (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03041-01 OD P

PERIOD COVERED
July 1, 1976 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Valve Ring Abscess in Active Infective Endocarditis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: E.N. Arnett	Staff Associate Sect. Pathol.	NHLI
OTHER: W.C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of 95 necropsy patients with active infective endocarditis (AIE) involving 128 native cardiac valves (aortic = 59, mitral = 48, tricuspid = 20, and pulmonic = 1) disclosed 27 patients with ring abscesses involving 30 valves. Comparison of the following parameters, showed significant ($p < 0.05$) differences between the 27 pateints with and the 68 patients without valve ring abscess: 1) infection of the aortic valve; 2) occurrence of valvular regurgitation of recent origin; 3) presence of pericarditis; 4) presence of high degree of atrioventricular block; and 5) short duration of symptoms leading to severe debility or death.

1151

PHS-6040
(12-75)

Project Description: Analysis of 95 necropsy patients with active infective endocarditis (AIE) involving 128 native cardiac valves (aortic = 59, mitral = 48, tricuspid = 20, and pulmonic = 1) disclosed 27 patients with ring abscesses involving 30 valves: the aortic valve ring was infected in 24 patients and only an atrioventricular valve ring (mitral in two, and tricuspid in one) in three patients. Comparison of the following parameters showed no significant differences between the 27 patients with and the 68 patients without ring abscess: age, sex, antibiotic treatment or length of treatment, status of the cardiac valve(s) before infection, and the kind of infecting organism. Comparison of the following parameters, however, showed significant ($p < 0.05$) differences between the 27 patients with and the 68 patients without valve ring abscess: 1) infection of the aortic valve; 2) occurrence of valvular regurgitation of recent origin; 3) presence of pericarditis; 4) presence of high degree of atrioventricular block; and 5) short duration of symptoms leading to severe debility or death. These five features, therefore, serve as clinical clues to the presence of valve ring abscess in patients with AIE.

Publications: Arnett, E.N., Roberts, W.C.: Valve Ring Abscess in Active Infective Endocarditis Frequency, Location, and Clues to Clinical Diagnosis from Study of 95 Necropsy Patients. Circulation (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03042-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Intravenous Injection on Talc-containing Drugs Intended for Oral Use

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. N. Arnett	Staff Associate Sect. Pathol. NHLI
OTHER:	W. E. Battle	Georgetown Medical Service, D.C. General, Washington, D.C.
	J. V. Russo	Washington Hospital Center, Washington, D.C.
	W. C. Roberts	Chief, Sect. Pathol. NHLI

COOPERATING UNITS (if any)

Department of Medicine, Georgetown University, District of Columbia Gen. Hospital, Washington, D.C. and Washington Hospital Center, Washington, D.C.

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and morphologic features are described in 2 patients known to have repeatedly injected intravenously talc-containing drugs intended for oral use. One patient developed severe pulmonary hypertension and the talc granulomas in him were located predominately within the pulmonary arteries. The second patient had normal pulmonary arterial pressures and the talc granulomas in him were located predominately in the pulmonary interstitium.

1153

PHS-6040
(12-75)

Project No. Z01 HL 03042-01 OD P

Project Description: Clinical and morphologic features are described in two patients known to have repeatedly injected intravenously talc-containing drugs intended for oral use. One patient developed severe pulmonary hypertension and the talc granulomas in him were located predominately within the pulmonary arteries. The second patient had normal pulmonary arterial pressures and the talc granulomas in him were located predominately in the pulmonary interstitium. Of 19 previously reported patients with pulmonary talc graulomas, 12 had morphologic evidence of pulmonary hypertension (in 3 of severe degree) and in each talc granulomas were located predominately within the pulmonary arteries. In those without signs of pulmonary hypertension, granulomas were located predominately in the pulmonary interstitium. Why there are differences in the distribution of the talc granulomas is unclear. It is clear, however, as demonstrated by one of our patients, that severe pulmonary hypertension may be a consequence of intravenous injection of drugs intended for oral use.

Publications:

Arnett, E.N., Battle, W.E., Russo, J.V., Roberts, W.C.: Intravenous Injection of Talc-containing Drugs Intended for Oral Use: A Cause of Pulmonary Granulomatosis and Pulmonary Hypertension. American Journal of Medicine Vol 60: 711-718, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 03043-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Atherosclerotic Narrowing of the Left Main Coronary Artery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	B. H. Bulkley	Staff Fellow, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Histologic sections of the left main (LM) and other three major coronary arteries were studied in 152 patients. The lumen of the LM in 35 patients was >75% narrowed; in thirty, 50-75%; and in 87, <50% narrowed. The patients with >75% narrowing were younger. Angina pectoris and hyperlipoproteinemia, specifically type II, were more common ($p < 0.02$) and acute transmural and healed subendocardial myocardial infarcts were less frequent ($p < 0.05$) in the patients with >75% LM narrowing than in those with <50% narrowing. Of the three other major coronary arteries, the average number narrowed in the patients with <50% LM narrowing, 2.4. Of the 35 patients with >75% luminal narrowing of each of the other three major coronary arteries.

1155

PHS-6040
(12-75)

Project Description: Histologic sections of the left main (LM) and other three major coronary arteries were studied in 152 patients. The lumen of the LM in 35 patients was > 75% narrowed; in thirty, 50-75%; and in 87, < 50% narrowed. The patients with > 75% narrowing were younger. Angina pectoris and hyperlipoproteinemia, specifically type II, were more common ($p \leq 0.02$) and acute transmural and healed subendocardial myocardial infarcts were less frequent ($p < 0.05$) in the patients with > 75% LM narrowing than in those with < 50% narrowing. Of the three other major coronary arteries, the average number narrowed in the patients with < 50% LM narrowing, 2.4. Of the 35 patients with > 75% LM narrowing, 33 had > 75% luminal narrowing of each of the other three major coronary arteries. Narrowing of the LM, therefore, indicates severe narrowing of usually all major coronary arteries.

Publications: Bulkley, B.H., Roberts, W.C.: Atherosclerotic Narrowing of the Left Main Coronary Artery: A Necropsy Analysis of 152 Patients with Fatal Coronary Heart Disease and Varying Degrees of Left Main Narrowing. Circulation 53: 823-828, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03044-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Congenital Obstructive Lesions Involving The Major Pulmonary Veins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D.L. Glancy Dept. Med. Hotel Dieu Hospital New Orleans, La.
OTHER: W.C. Roberts, Chief, Sect. Pathol. NHLI

COOPERATING UNITS (if any)

Cardiology Branch NHLI

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This report summarizes congenital causes of pulmonary venous hypertension. Descriptions of both clinical and morphologic features of obstruction of congenital obstructions of the major pulmonary veins, including partial and total anamolous pulmonary venous connection, cor triatriatum, mitral valve atresia, supravalvular left atrial ring, and congenital mitral stenosis are illustrated. In contrast to acquired lesions which interfere with left ventricular filling, congenital lesions are quite rare. This paper summarizes a large personal experience with the congenital lesions which lead to pulmonary venous hypertension.

1157

PHS-6040
(12-75)

Project Description: This report summarizes congenital causes of pulmonary venous hypertension. Descriptions of both clinical and morphologic features of obstruction of congenital obstructions of the major pulmonary venis, including partial and total anamolous pulmonary venous connection, cor triatriatum, mitral valve atresia, supravalvular left atrial ring, and congenital mitral stenosis are illustrated. In contrast to acquired lesions which interfere with left ventricular filling, congenital lesions are quite rare. This paper summarizes a large personal experience with the congenital lesions which lead to pulmonary hypertension.

Publications: Glancy, D.L., Roberts, W.C.: Congenital Obstructive Lesions Involving The Major Pulmonary Veins, Left Atrium Or Mitral Valve: A Clinico-Morphologic Survey. Catheterization & Cardiovascularal Division (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03045-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Pathologic Anatomy of the Cardiomyopathies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	V. J. Ferrans	Visiting Scientist, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

None

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SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This presentation summarized necropsy observations in patients with three types of cardiomyopathy: idiopathic, infiltrative, and endomyocardial disease.

1159

PHS-6040
(12-75)

Project Description: This presentation summarized necropsy observations in patients with three types of cardiomyopathy: idiopathic, infiltrative, and endomyocardial disease. The idiopathic variety is subdivided into two types depending on the size of the ventricular cavity. In the dilated ventricular type the left ventricular wall is frequently less than 1.5 cm. thick, intracardiac thrombi are common, the atrioventricular valve rings usually are mildly dilated, and focal myocardial and endocardial scars are common. In the nondilated type (hypertrophic cardiomyopathy), the ventricular septum is usually thicker than the left ventricular free wall, which also is thick (>1.5 cm.). When the septum is similar in thickness to the left ventricular free wall (symmetric), left ventricular outflow obstruction does not occur. When the septum is thicker than the left ventricular free wall (asymmetric), left or right ventricular outflow obstruction may or may not be present. The orientation of myocardial fibers one to another in the ventricular septum in the nondilated (hypertrophic) type is abnormal, whereas it is normal in the dilated ventricular type. Intracardiac thrombi are rare and atrioventricular valve rings are never dilated in the nondilated type of idiopathic cardiomegaly.

The infiltrative types of cardiomyopathies include iron, calcium, lipids, mucopolysaccharides, granulomas, amyloid, and neoplasms. The first four usually are located within myocardial cells and the latter three, between myocardial cells. It is probable that all these myocardial infiltrates are capable of producing cardiac dysfunction, primarily on a restrictive basis.

Endomyocardial disease may or may not be associated with eosinophilia. When the latter occurs, the eosinophils are structurally normal. Death is related to congestive cardiac failure. This category in actuality also is idiopathic.

Publications: Roberts, W.C. and Ferrans, V.J.: Pathologic Anatomy of the Cardiomyopathies. Idiopathic Dilated and Hypertrophic Types, Infiltrative Types, and Endomyocardial Disease with and without Eosinophilia. Human Pathol. 6:287-342, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03046-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Dilatation of the Mitral Anulus: A Rare Cause of Mitral Regurgitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	B. H. Bulkley	Staff Fellow, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
None

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Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

To confirm or deny the thesis that mitral anular dilatation is a cause of mitral regurgitation, this anulus was measured in 102 patients, 24 of whom had normal hearts and 78 of whom had dilated left ventricles, some associated with mitral regurgitation, others not. Dilatation of the mitral anulus is a rare cause of mitral regurgitation and that abnormality of the fibrous skeleton of the heart is necessary for the mitral anulus to dilate enough to cause mitral regurgitation.

1161

PHS-6040
(12-75)

Project No. Z01 HL 03046-01 OD P

Project Description: To confirm or deny the thesis that mitral anular dilatation is a cause of mitral regurgitation, this anulus was measured in 102 patients, 24 of whom had normal hearts and 78 of whom had dilated left ventricles, some associated with mitral regurgitation, others not. The circumference of the mitral anulus in the 24 patients with normal hearts averaged 9 cm (range 7 to 11 cm); in 24 patients with idiopathic cardiomyopathy of the ventricular dilated type, both with (12 patients) and without (12 patients) mild to moderate regurgitation, 11 cm (range 10 to 14 cm); and in 31 patients with severe mitral regurgitation, 12 cm (range 8 to 18 cm). Among the latter 31 patients with severe mitral regurgitation, the anulus was only mildly dilated in the 15 with rheumatic disease (avg 11 cm) and in the six with ruptured chordae tendineae on previously normal valves (avg 10 cm); in contrast, in the 10 patients with floppy mitral valves with or without the Marfan syndrome the mitral anulus was greatly dilated (avg 15.5 cm). Thus, only in patients with floppy valves with or without the Marfan syndrome or both does anular dilatation by itself appear great enough to cause mitral regurgitation. It appears, therefore, that dilatation of the mitral anulus is a rare cause of mitral regurgitation and that abnormality of the fibrous skeleton of the heart is necessary for the mitral anulus to dilate enough to cause mitral regurgitation.

Publications: Bulkley, B.H. and Roberts, W.C.: Dilatation of the Mitral Anulus: A Rare Cause of Mitral Regurgitation. Am.J. Med. 59:457-463, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03047-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Heterografts as Aortocoronary Bypass Conduits in Human Beings

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. C. Roberts	Chief, Sect. Pathol.	NHLI
OTHER:	B. H. Bulkley	Staff Fellow, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

None

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Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and necropsy observations are described in a man in whom 2 sheep carotid arterial heterografts were inserted as aortocoronary bypass conduits 30 months before death, and in whom a canine saphenous vein heterograft was inserted several hours before death. Examination of previous reports describing heterografts for vascular reconstruction or bypass in both man and experimental animals and observations in our patients suggest that heterografts are unsatisfactory as aortocoronary bypass conduits.

1163

PHS-6040
(12-75)

Project Description: Clinical and necropsy observations are described in a man in whom 2 sheep carotid arterial heterografts were inserted as aorto-coronary bypass conduits 30 months before death, and in whom a canine saphenous vein heterograft was inserted several hours before death. All 3 grafts failed. The lumens of the sheep carotid arterial heterografts had closed by the time of catheterization 6 months after operation; at necropsy both grafts were totally occluded at their coronary and arotic ends and their media and adventitia were infiltrated by granulomatous inflammatory cells. Little information is available regarding the use of heterografts as coronary bypass conduits. Examination of previous reports describing heterografts for vascular reconstruction or bypass in both man and experimental animals and observations in our patient suggest that heterografts are unsatisfactory as aortocoronary bypass conduits.

Insertion of a conduit between the ascending aorta or one of its branches and a coronary artery is now a common form of treatment in patients with severe symptomatic coronary atherosclerosis. The vessel most commonly utilized for the conduit is an autogenous saphenous vein and the next most frequent, an autogenous mammary artery. A suitable substitute for these autogenous vessels in the aortocoronary position would be useful. Synthetic grafts small enough to anastomose to the coronary artery have been unsatisfactory because of clotting problems. Attention recently has been refocused on vascular heterografts as possible conduits between the aorta and the coronary artery. Stimulated by study at necropsy of a patient given two types of heterografts as aortocoronary conduits, we reviewed previous utilization of heterografts in man. Study of these previous reports and observations in our patient are summarized in this report.

Publications: Bulkley, B. H., and Roberts, W. C.: Heterografts as Aortocoronary Bypass Conduits in Human Beings. Am. J. Cardiol. 36:823-828, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 03048-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Coronary Heart Disease after Mediastinal Irradiation for Hodgkin's Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. A. McReynolds	Staff Associate, Sect. Pathol.	NHLI
OTHER:	G. L. Gold	Consultant, Div. Cancer Treatment	NCI
	W. C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)
Division of Cancer Treatment, National Cancer Institute

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Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

Attention is called to the development of coronary heart disease in two patients several years after they received mediastinal irradiation for Hodgkin's disease. One patient, a 33-year-old man, died suddenly 8 years after irradiation; necropsy disclosed marked narrowing of all three major coronary arteries. Observations in previously described patients with coronary heart disease almost surely induced by therapeutic irradiation for Hodgkin's disease are reviewed.

1165

PHS-6040
(12-75)

Project No. Z01 HL 03048-01 OD P

Project Description: Attention is called to the development of coronary heart disease in two patients several years after they received mediastinal irradiation for Hodgkin's disease. One patient, a 33-year-old man, died suddenly eight years after irradiation; necropsy disclosed marked narrowing of all three major coronary arteries. In addition to severe intima fibrous thickening, there also was considerable adventitial scarring of the coronary arteries. This type of coroanry sclerosis is different from that seen in the usual patient with coronary heart disease. The second patient, a 42-year-old man, had an acute myocardial infarction on two occasions, the first six years after mediastinal irradiation. Observations in previously described patients with coronary heart disease almost surely induced by therapeutic irradiation for Hodgkin's disease are reviewed.

Publications: McReynolds, R.A., Gold, G.L., and Roberts, W.C.: Coronary Heart Disease after Mediastinal Irradiation for Hodgkin's Disease. Am.J. Med. 60:39-44, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 03049-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Active Infective Endocarditis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.N. Arnett	Staff Associate, Sect. Pathol.	NHLI
OTHER:	W.C. Roberts	Chief, Sect. Pathol.	NHLI

COOPERATING UNITS (if any)

None

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Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	0

SUMMARY OF WORK (200 words or less - underline keywords)

This large monograph summarizes, primarily, morphologic observations in 23 patients with infective endocarditis involving right-sided cardiac valves with or without involvement of left-sided valves, 74 patients with infective endocarditis limited ot left-sided cardiac valves, 22 patients with prosthetic valve endocarditis, 4 patients with infective endocarditis after valvuloplasty operations, 6 patients with infective endocarditis complicating congenital cardiac disease with a shunt, and 8 with infective endocarditis limited to mural endocardium. It is clear the in the past decade that infective endocarditis is far more common on previously normal valves both anatomically and functionally than on previously abnormal valves.

1167

PHS-6040
(12-75)

Project No. ZOL HL 03049-01 OD P

Project Description: This large monograph summarizes, primarily, morphologic observations in 23 patients with infective endocarditis involving right-sided cardiac valves with or without involvement of left-sided valves, 74 patients with infective endocarditis limited to left-sided cardiac valves, 22 patients with prosthetic valve endocarditis, 4 patients with infective endocarditis after valvulotomy or valvuloplasty operations, 6 patients with infective endocarditis complicating congenital cardiac disease with a shunt, and 8 with infective endocarditis limited to mural endocardium. It is clear that in the past decade that infective endocarditis is far more common on previously normal valves both anatomically and functionally than on previously abnormal valves.

Publications: This monograph is accepted as a chapter in a book to be published by Grune & Stratton in late 1976. The book is entitled INFECTIVE ENDOCARDITIS.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03050-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Skeletal Muscle Ultrastructure in Selenium-Vitamin E Deficiency in Chicks

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John F. Van Vleet, Purdue University School of Veterinary Medicine
OTHER: Victor J. Ferrans, Visiting Scientist, Sect. Pathol., NHLI

COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine, Lafayette, Indiana

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Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.1	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This study describes the histologic and electron microscopic features of the skeletal muscle degeneration and exudative diathesis that develop in chicks deficient in selenium and vitamin E.

1169

PHS-6040
(12-75)

Project Description: Chicks fed a semisynthetic basal diet deficient in selenium and vitamin E for 14 to 22 days developed skeletal myodegeneration and exudative diathesis. Chicks fed the basal diet supplemented with either 0.2 ppm selenium as selenite or 100 I.U. α tocopherol acetate/kg were protected from deficiency disease, but chicks fed the basal diet plus 0.4% *l*-cystine were not protected. Pectoral muscles of deficient chicks were red and edematous. Light and electron microscopic study of affected muscles revealed fibers with hyaline and granular degeneration. In hyalinized fibers, the initial ultrastructural alterations were increased density of the sarcoplasm and myofibrils, dilatation of sarcoplasmic reticulum, formation of subsarcolemmal vacuoles, and disruption of mitochondrial membranes. In later stages, alterations in these fibers included myofibrillar disruption and lysis, nuclear pyknosis and lysis, disruption of the plasma membrane with persistence of basal lamina and scattered adhering satellite cells, and eventual invasion by macrophages. In fibers with granular degeneration, the ultrastructural findings included decreased density of the sarcoplasm, prominent mitochondrial swelling and distortion, and multiple foci of myofibrillar lysis that eventually coalesced to produce generalized lysis. Prominent vascular lesions associated with exudative diathesis were present in the degenerated muscle but were not considered to precede the development of the fiber alterations. Affected vessels had endothelial cells with mitochondrial damage and accumulations of cytoplasmic dense bodies, and areas of endothelial disruption with adhering fibrin thrombi. The results of these studies support the concept that selenium and vitamin E play a role in protecting cellular membranes from lipoperoxidation.

Publications: Van Vleet, J.F. and Ferrans, V.J.: Ultrastructural changes in skeletal muscle of selenium-vitamin E deficient chicks. Am J Vet Res (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03051-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976.

TITLE OF PROJECT (80 characters or less)

Morphology of cardiac rhabdomyomas.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John J. Fenoglio, Div. of Cardiovascular Pathology, AFIP, Washington, DC
OTHER: Victor J. Ferrans, Vis. Scient. Sect. Pathol., NHLI
Hugh A. McAllister, Div. of Cardiovascular Pathol., AFIP, Washington, DC

COOPERATING UNITS (if any)
Division of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, DC.

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Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and pathologic features of cardiac rhabdomyomas in 36 patients are described , including 7 patients in whom the tumors were examined by electron microscopy. It is concluded from these studies that rhabdomyomas of the heart are definitely derived from cardiac muscle cells and probably represent hamartomas rather than true tumors.

1171

Project No. Z01 HL 03051-01 OD P

Project Description: Clinical and pathologic features of cardiac rhabdomyomas from 36 patients are described. Of these tumors, 90% were multiple and 78% occurred in children under one year of age. In 56% of the patients at least one of the tumor masses was intracavitary and in 42% it obstructed one or more of the cardiac valves. The tumors occurred with equal frequency in the right and left ventricles and in subepicardial and subendocardial locations. Ultrastructural study of tumors from 7 patients revealed similar features: paucity of myofibrils, which were arranged either parallel to the cell surfaces or in strands radiating toward the center; randomly located intercellular junctions, which had specialized components similar to those of intercalated discs of normal myocardium; and numerous leptofibrils, which were arranged either peripherally or in centrally located, spiralling clusters. It is concluded that cardiac rhabdomyomas are derived from muscle cells and probably represent hamartomas rather than true tumors.

Publications: Fenoglio, J. J., McAllister, H. A. and Ferrans, V. J.: Cardiac rhabdomyoma: a clinicopathologic and electron microscopic study. Am. J. Cardiol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03052-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Skeletal Muscle Ultrastructure in Selenium-Vitamin E Deficiency in Pigs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John F. Van Vleet, Purdue University School of Veterinary Medicine

OTHER: Victor J. Ferrans Visiting Scientist Sect. Pathol. NHLI

George R. Ruth Purdue University School of Veterinary Medicine

COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine, Lafayette, Indiana

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Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This study describes histologic and ultrastructural studies of skeletal muscle lesions present in pigs fed a semisynthetic diet deficient in selenium and vitamin E for 13 to 59 days. Major alterations observed consisted of hyaline degeneration of the muscle cells, with subsequent macrophagic invasion and muscle cell regeneration by myoblastic proliferation, fusion and differentiation into fibers with mature myofibrils.

1173

PHS-6040
(12-75)

Project Description: Light and electron microscopic studies were made of the lesions which developed in the skeletal muscles of 24 of 38 young growing pigs fed a semisynthetic diet deficient in selenium and vitamin E for 13 to 59 days. The major alterations in injured fibers progressed from hyaline degeneration, with subsequent macrophagic invasion and phagocytosis of disrupted sarcoplasm, to muscle fiber regeneration by myoblastic proliferation, fusion and differentiation into fibers with mature myofibrils.

The earliest electron microscopic alterations observed were myofibrillar lysis and disruption, with thick filaments persisting longest. Disruption of mitochondria, sarcoplasmic reticulum and plasma membranes was seen in fibers with myofibrillar alterations. The basal lamina of the sarcolemma remained after destruction of the enclosed sarcoplasm and served as a scaffold for subsequent regeneration. Stages of regeneration that were observed included myoblastic proliferation, fusion into cords and myotubes, and finally, fibrillogenesis to restore contractile material.

The sequence of structural alterations found in this study was discussed in terms of current theories of the biochemical role of selenium and vitamin E in maintaining cellular integrity.

Publications: Van Vleet, J. F., Ruth, G. R. and Ferrans, V. J.: Ultrastructural alterations in skeletal muscle of selenium-vitamin E deficient pigs. Am. J. Vet. Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03053-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Heart in the Hurler Syndrome.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Vicente G. Renteria	Guest Worker	Sect. Pathol., NHLI
OTHER:	Victor J. Ferrans	Vis. Scient.	Sect. Pathol., NHLI
	William C. Roberts	Chief	Sect. Pathol., NHLI

COOPERATING UNITS (if any)

None

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Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

Cardiovascular lesions in 5 patients with the Hurler syndrome involved the coronary arteries, the 4 cardiac valves, the mural endocardium, the myocardium and the aorta. These sites contained large, clear cells with deposits of acid mucopolysaccharides; in addition, cardiac muscle cells and smooth muscle cells contained deposits of glycolipid material. These changes led to valvular deformities and to extremely severe narrowing of the coronary arteries.

1175

Project Description: Clinical and morphologic features of the cardiovascular system are described in five necropsy patients with the Hurler syndrome. In all five patients the coronary arteries, four cardiac valves, mural endocardium of all four chambers, myocardial walls and aorta were affected in a characteristic manner. All of these sites contained large clear cells known as Hurler cells (readily visible by light microscopy). In addition, granular cells were observed in semithin (1 micron) sections and by electron microscopy in the coronary arteries, atrioventricular valves and in myocardial interstitium. These latter cells appear to produce collagen in an abnormal way and are probably responsible for the heavy deposits of collagen in the cardiovascular system of patients with the Hurler syndrome. In the cardiac muscle cells, smooth muscle cells of the coronary arteries and in fibroblasts, wherever located, deposits of acid mucopolysaccharides (AMP) and glycolipids usually also were observed. The AMP deposits were observed easily by light microscopy except in the cardiac muscle cells and in them they were seen only by electron microscopy. The glycolipid deposits, observed only by examination of 1 micron thick sections or by electron microscopy, have not been observed previously in coronary arteries or in myocardial cells.

The infiltration into the heart by the above cells and deposits in all 5 patients resulted in severe narrowing of the extramural coronary arteries, considerable thickening of the cardiac valves (the left-sided ones more than the right-sided ones), generalized thickening of mural endocardium, and "stiffening" of the myocardial walls. Thus, the cardiovascular lesions in the Hurler syndrome are specific and life-threatening.

Publications: Renteria, V.G., Ferrans, V.J. and Roberts, W.C.: The heart in the Hurler syndrome: gross, histologic and ultrastructural observations in five necropsy patients. Am J Cardiol (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 03054-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)
Sarcoplasmic reticulum in atrial myocardium in mitral valve disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI; Klaus-Ulrich Thiedemann Guest Worker Sect. Pathol. NHLI
OTHER: Victor J. Ferrans Vis. Scient. Sect. Pathol. NHLI

COOPERATING UNITS (if any)

None

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Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.10	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This paper describes ultrastructural abnormalities observed in sarcoplasmic reticulum in atrial myocardium of patients with mitral valvular disease. These abnormalities consist of various types of proliferation and of aggregation of the tubules and cisterns of sarcoplasmic reticulum.

1177

PHS-6040
(12-75)

Project Description: Alterations observed in the sarcoplasmic reticulum of muscle cells in left and right atrial myocardium from 10 patients with mitral valvular disease consisted of: 1) proliferation of rough-surfaced endoplasmic reticulum, which formed large cisterns in perinuclear areas of hypertrophied cells and was considered indicative of increased protein synthesis; 2) proliferation of free sarcoplasmic reticulum, a change that occurred in degenerated cells and appeared to be related to loss of contractile elements; 3) two types of aggregates of tubules of free SR: one type was associated with abnormal Z-band material and was found only in cells showing loss of myofibrils and proliferation of free SR, whereas the other was not associated with either of these changes and occurred in less severely altered cells; and 4) proliferation and enlargement of cisterns of extended junctional sarcoplasmic reticulum, which formed two distinct types of complexes: the first of these consisted of large, convoluted (type A) cisterns that were wide (550 to 650 Å in thickness) and did not have a central dense lamina; the second was composed of stacks of concentric or parallel (type B) cisterns that were narrower (220 to 300 Å in thickness), had a central dense lamina and were separated from one another by layers of glycogen granules. The formation of these complexes of cisterns was regarded as an extreme form of overdevelopment of extended junctional sarcoplasmic reticulum in atrial muscle cells.

Publications: Thiedemann, K.-U. and Ferrans, V.J.: Ultrastructure of sarcoplasmic reticulum in atrial myocardium of patients with mitral valvular disease. Am J Pathol 83: 1-38, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03055-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Intracellular collagen in heart valves of patients with the Hurler syndrome.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Vicente G. Renteria Guest Worker Sect. Pathol., NHLI
OTHER: Victor J. Ferrans Vis. Scient. Sect. Pathol., NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.1	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

Collagen fibrils were found within cytoplasmic vacuoles in degenerated fibroblasts in cardiac valves of patients with the Hurler syndrome. An extensive review was made of the literature on intracellular collagen.

1179

PHS-6040
(12-75)

Project No. Z01 HL 03055-01 OD P

Project Description: Ultrastructural study of thickened mitral and tricuspid valves from 3 patients with the Hurler syndrome disclosed collagen fibrils located within membrane-bound cytoplasmic dense bodies in small granular cells. These cells, which differ morphologically from the clear cells containing mucopolysaccharide deposits, are considered to be fibroblasts in advanced stages of degeneration. It is postulated that the elevated concentrations of dermatan sulfate in Hurler's disease lead to an abnormally high synthesis of collagen and to its polymerization in intracellular loci.

Publications: Renteria, V.G. and Ferrans, V.J.: Intracellular collagen fibrils in cardiac valves of patients with the Hurler syndrome. Lab Invest 34: 263-272, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03056-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976.

TITLE OF PROJECT (80 characters or less)

Sarcolemmal alterations in cardiac hypertrophy and degeneration.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Vis. Scient.	Sect. Pathol.	NHLI
OTHER:	L. Maximilian Buja	Staff Fellow	Sect. Pathol.	NHLI
	Barry J. Maron	Senior Invest.	CB	NHLI

COOPERATING UNITS (if any)

Cardiology Branch, NHLI

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This paper presents a comprehensive review of alterations which occur in non-specialized and specialized (intercellular junctions and T-tubules) regions of the sarcolemma of cardiac muscle cells in hypertrophy and degeneration.

1181

PHS-6040
(12-75)

Project Description: Sarcolemmal alterations were studied in hypertrophied and/or degenerated ventricular muscle cells from: 1) patients with hypertrophic cardiomyopathy, congestive cardiomyopathy of various causes, aortic valvular disease, or congenital heart diseases associated with obstruction to right ventricular outflow; 2) patients with various neoplasms treated with anthracycline drugs; 3) rabbits given large doses of anthracyclines, and 4) dogs subjected to anoxic cardiac arrest during total cardiopulmonary bypass. Sarcolemmal changes in hypertrophied, nondegenerated cardiac muscle cells consisted of: 1) increase in the area and in the degree of irregularity of cell surfaces, including intercalated discs and T tubules; 2) dilation of T tubules, and 3) formation of large numbers of multiple intercalated discs. Moderately degenerated cardiac muscle cells, with or without associated hypertrophy, showed sarcolemmal changes characterized by: 1) dilation and decreased numbers of T tubules; 2) marked surface irregularity; and 3) dissociation of intercalated discs, associated with development of vesicles in widened disc interspaces, and of variably complex junctional structures formed between two parts of the plasma membrane of the same muscle cell (intracytoplasmic junctions). Sarcolemmal alterations in cells with end-stage degeneration were associated with loss of myofibrils and consisted of: 1) disappearance of T tubules; 2) loss of junctional contacts with adjacent cells; 3) marked surface irregularity; 4) formation of intracytoplasmic junctions, and 5) thickening of basement membranes. These observations show that remodeling of the cell surfaces is an important feature of the cellular responses to the stimuli of hypertrophy or degeneration.

Publications: Ferrans, V.J., Buja, L.M., and Maron, B.J.: Sarcolemmal alterations in cardiac hypertrophy and degeneration. In Roy, P.-E. and Dhalla, N.S. (Eds.): The Sarcolemma, Recent Advances in Studies on Cardiac Structure and Metabolism. Baltimore, University Park Press, 1976, Volume No. 9, pp. 395-419.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03057-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Myocardial injury produced by antineoplastic drugs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: L. Maximilian Buja Staff Fellow Sect. Pathol., NHLI
OTHER: Victor J. Ferrans Vis. Scient. Sect. Pathol., NHLI

COOPERATING UNITS (if any)
None

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.1	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This paper describes cardiac morphologic changes induced by the use of high doses of anthracycline types of antineoplastic agents, either alone or in combination with high doses of cyclophosphamide.

1183

PHS-6040
(12-75)

Project Description: Histologic and ultrastructural studies were performed on the hearts of 16 patients with fatal leukemias or solid tumors. Multifocal myocardial degenerative lesions, characterized by alterations in contractile elements, mitochondria, and nuclei, were found in 12 patients treated with high total doses of anthracyclines, but not in 3 patients who did not receive such therapy. One patient who received a high dose of cyclophosphamide as well as a high total dose of daunorubicin exhibited extensive hemorrhagic myocardial necrosis and multifocal degenerative lesions. This study documents the cardiotoxic effects of anthracyclines and cyclophosphamide.

Publications: Buja, L.M. and Ferrans, V.J.: Myocardial Injury Produced by Antineoplastic Drugs. In Fleckenstein A. and Rona, G. (Eds.): Pathophysiology and Morphology of Myocardial Cell Alteration, Recent Advances in Studies on Cardiac Structure and Metabolism. Baltimore, University Park Press, 1975, Volume No. 6, pp. 487-497

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 03058-01 OD P

PERIOD COVERED

July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Myofibrillar abnormalities following cardiac muscle cell injury

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Visiting Scientist,	Sect. Pathol. NHLI
OTHER:	L. Maximilian Buja	Staff Fellow	Sect. Pathol. NHLI
	Barry J. Maron	Senior Invest.	Cardiology Br. NHLI

COOPERATING UNITS (if any)

Cardiology Branch, NHLI

LAB/BRANCH

ODIR

SECTION

Section of Pathology

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This paper presents a review of morphological alterations which occur in myofibrils of cardiac muscle cells in various types of experimentally induced and spontaneously occurring injury.

1185

PHS-6040
(12-75)

Project Description: A review is presented of morphological aspects of myofibrillar alterations which occur in cardiac muscle cells following various types of injury. Emphasis is placed on the association between specific patterns of alterations involving the structure or the orientation of myofibrils and diverse forms of myocardial injury.

The following conclusions are drawn from the observations discussed in this review: (1) Selective alterations involving thick filaments, thin filaments, and Z-bands occur under different conditions of damage. (2) Aggregations of Z-band material are present in cells undergoing atrophy, degeneration, or hypertrophy. (3) Maintenance of normal sarcomeric structure probably requires a state of equilibrium of mechanical and biochemical forces in the cell. (4) Morphological features of early stages of necrosis are determined by the specific conditions under which necrosis occurs. The extent to which tissue is perfused during the development of irreversible injury is critical in this respect. (5) Alterations in myofibrillar orientation are associated with unusual or abnormal patterns of mechanical forces, and are a prominent feature of hypertrophic cardiomyopathy.

Publications: Ferrans, V.J., Buja, L.M. and Maron, B.J.: Myofibrillar abnormalities following cardiac muscle cell injury. In Fleckenstein, A. and Rona G. (Eds.): Pathophysiology and Morphology of Myocardial Cell Alterations, Recent Advances in Studies on Cardiac Structure and Metabolism. Baltimore, University Park Press, 1975, Volume No. 6, pp. 367-382

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03059-01 OD P

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Infantile cardiomyopathy with histiocytoid change.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Vis. Scient. Sect. Pathol., NHLI
OTHER: Hugh A.McAllister, Chief, Div. of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, DC
Wolfgang H. Haese, Department of Pathology, Saint Luke's Episcopal Hospital, Houston, Texas.

COOPERATING UNITS (if any)
Division of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, DC, and Department of Pathology, Saint Luke's Episcopal Hospital, Houston, Texas.

LAB/BRANCH
ODIR

SECTION
Section of Pathology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.30	0.15	0.15

SUMMARY OF WORK (200 words or less - underline keywords)

This paper describes clinical and pathologic features of a group of patients with a clinicopathologic syndrome termed "infantile cardiomyopathy with histiocytoid change in cardiac muscle cells". This syndrome is manifested clinically by recurrent cardiac arrhythmias and sudden death, and pathologically by a distinctive type of focal degeneration and histiocyte-like appearance of the muscle cells. This syndrome has been found to occur only in children aged 6 to 24 months, and its etiology is unknown.

1187

Project No. ZO1 HL 03059-01 OD P

Project Description: Clinical and pathologic findings are presented in 14 patients (6 newly reported and 8 described previously, all children ranging in age from 6 to 24 months) with a clinicopathologic syndrome termed "infantile cardiomyopathy with histiocytoid change in cardiac muscle cells". This syndrome is manifested clinically by severe, eventually fatal cardiac arrhythmias, and is characterized pathologically by cardiac hypertrophy and by a distinctive type of focal degeneration of the muscle cells, which lose their myofibrils, undergo marked mitochondrial hyperplasia, become rounded in shape and enlarged, and resemble histiocytes. Evidence is presented to support the conclusions that these manifestations are those of a cardiomyopathy, that cardiac hypertrophy preceded the onset of the clinical features, that the focal degeneration is likely to be a cause rather than a consequence of the arrhythmias, and that the latter develop only in the late stages of the disorder. The etiology of this cardiomyopathy remains unclear.

Publications: Ferrans, V. J., McAllister, H. A. and Haese, W. H. : Infantile cardiomyopathy with histiocytoid change in cardiac muscle cells. Report of six patients. Circulation 53: 708-719, 1976

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30,1976

The primary interest of the Section on Theoretical Biophysics is the theory of transport processes in biological systems, with particular reference to problems in cardiovascular, renal, and membrane physiology. The section is concerned both with the formulation of theoretical models and with the development of mathematical and computational methods for their analysis. Currently much of the research in the section centers on the mechanism of urine formation in the mammalian kidney.

Mathematical Models of the Kidney

During the past year in our extension of our earlier work on kidney models, we have developed a multinephron multisolute model of the whole kidney. This model takes account of the cortical and medullary distribution of nephrons and capillaries and includes salt, water, and urea transport, and both hydrostatic and oncotic pressure. With this model it should be possible to analyze several aspects of renal function such as the effect of urea cycling on passive salt transport out of thin ascending limb of Henle, and the effects of changes in the distribution of blood flow between cortex and medulla.

We also developed a multinephron central core model of the renal medulla and extended our earlier work on single nephron models of the kidney to include effects of protein leak in cortical and medullary capillaries.

Work was also begun on models of epithelial transport. This work is directed toward both the analysis of isolated tubule experiments and the inclusion of a suitable model of proximal tubule into the whole kidney models.

In all of these computer simulations the need for a steady effort to improve numerical methods has been apparent. Toward this end we have continued our cooperative and collaborative programs with mathematicians at the University of Maryland, Louisiana Polytechnic Institute, and SUNY, Stony Brook, N. Y. We also have spent considerable time in the section comparing the stability and efficiency of various algorithms. In order to promote an exchange of information on these problems we organized two sessions on numerical methods in kidney modeling as part of the 1976 Summer Computer Simulation Conference, which will take place in Washington, D. C.

A review of the past, present, and future of kidney modeling was carried out as part of the NIAMDD survey of research needs in nephrology. From this survey it seemed clear that models of the mammalian kidney have reached a level of sophistication such that realistic simulation of several aspects of renal function is now possible and simulation of additional features should be possible in the near future. These models should provide the basis for the estimation of transmembrane transport parameters from data on whole kidney function. If this goal can be reached, it will give new interpretations of both normal and abnormal renal function.

1189

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03201-16 STB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)
Mathematical Theory of Renal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLI
OTHER:	R. Mejia	Mathematician	OD NHLI
	B. Kellogg	IFDAM, Univ. of Md.	
	K. Crump	Prof. Louisiana Polytech. Inst. Ruston	
	J. Garner	Prof. Louisiana Polytech. Inst. Ruston	

COOPERATING UNITS (if any)
Math. Research Branch, NIAMDD; IFDAM, Univ. of Maryland; Louisiana Polytechnic Institute, Ruston, La.

LAB/BRANCH

SECTION
Section on Theoretical Biophysics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.6	.2

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop the general theory of transport and flow processes taking place in the kidney. Aims of current work include (1) thermodynamic analysis of flow processes, (2) the qualitative analysis of equations describing kidney models, (3) the development of analytical solutions of kidney models, and (4) the general theoretical analysis of numerical methods.

1191

PHS-6040
(12-75)

Project Description:

Objectives: The primary purpose of this project is to develop the general theory of the transport and flow taking place in the kidney. This includes the thermodynamic analysis of flow processes; the qualitative analysis of equations describing kidney models, the development of analytical solutions of kidney models, and the general theoretical analysis of numerical methods.

Specific Findings: (1) The canonical mass balance equation derived for the central core model of the renal medulla has been extended to medullary models in which an arbitrary assemblage of renal tubules and vascular capillaries exchange with each other both directly and via the medullary interstitium and in which not all of the vascular loops or loops of Henle extend to the papilla.

Specifically it has been found that if descending limbs of Henle and descending vasa recta enter the medulla at approximately plasma osmolality, the concentration ratio is given by

$$r = 1 \ /[1 - f_T \ (1 - f_U)(1 - f_W)],$$

where f_T is fractional solute transport out of AHL, f_U is fractional urine flow, and f_W is fractional dissipation; f_W is a measure of the solute returned to the systemic circulation without its isotonic complement of water.

It can be shown that

$$f_W \equiv W/[T_{2M} - T_{2V} \, C_{pM} + F_{3V} \, (0) \, \Delta \, C_{3M}],$$

and

$$W = (T_{1M} + T_{2M} + T_{3M}) - (T_{1V} + T_{2V} + T_{3V}) \, C_{pM},$$

where T_{1M}, T_{2M}, T_{3M} are total solute transport out of DHL's, AHL's and CD's; T_{1V}, T_{2V}, T_{3V} are total volume transport; C_{pM} is plasma osmolality; $F_{3V}(0)$ is entering CD flow; and $\Delta \, C_{3M}$ is the amount by which average entering CD concentration differs from plasma osmolality.

Thus, W/C_{pM} is the free water generated by the medulla, and the efficiency of the vascular exchanger is W/T_2' where T_2' is the effective AHL source.

The above relations are formally identical with those derived previously for the single nephron central core model of the renal medulla.

(2) Work on existence theory (Kellogg, Contract # HI 52900) has continued. In general we seek a theorem which guarantees, under very general conditions, the existence of solutions of the renal flow equations. It is likely that a key ingredient in the proof of an existence theorem is an a priori bound on a solution, if any, of the equations. (For certain parameter choices it can be shown that no solutions exist.)

(3) Singular perturbation problems arise from renal network equations by allowing certain membrane permeabilities to tend to infinity. We have started a study of these problems. One of the aims of the research is to obtain either error estimates or an asymptotic anyalsis of the solution in inverse powers of the permeability,(with Kellogg).

(4) Work on analytical solutions of time dependent equations of countercurrent systems with only solute exchange, and work on solution of such systems by numerical inversion of Laplace transform has continued. Earlier work has been written up for publication. One result of this work has been the first rigorous analysis of the classic Kuhn countercurrent multiplier. Although "multiplication" in a very loose sense takes place, the "single effect" varies both temporally and spatially. The actual behavior, now primarily of historical interest, differs considerably from the cartoons found in many medical text books (with J. Garner and K. Crump).

(5) A review of mathematical models of the kidney and its subsystems was carried out as part of the work of the survey by NIAMDD to identify research needs in nephrology. This review considered both the foundations of kidney models: conservation concepts, the phenomenology of epithelial transport, pressure flow relations, the general equations for kidney models, and mathematical methods of solving the equations. In so far as possible these concepts were analyzed in relation to published work on kidney models.

After this overview, an attempt was made to identify some of the main problems for current and future research. These separate into methodological or mathematical problems, and into the application of models to physiological problems. These general problems are discussed in relation to the work of the section in the narrative summary and will not be repeated here.

Significance to biomedical research:

This work gives conceptual insight into renal function and provides the basis for quantitative models of renal function.

Proposed Course:

We plan to continue work in the following general areas: 1) The general theoretical analysis of the non-linear partial and ordinary differential equations occurring in mathematical models of the kidney.

2) The development of methods of parameter analyses for mathematical models of the kidney.

Publications:

*Kellogg, B., Uniqueness in the Schauder Fixed point theorem, Technical Note BN-822, IFDAM, University of Maryland.

*Kellogg, B., Oncotic flow in a tube with stagnant points, Technical Note BN-818, IFDAM, University of Maryland.

*Kellogg, B. A Priori bounds for renal network flows. Proceedings of the 1976 Summer Simulation Conference, (In press).

*Supported by NHLI under Contract HI 52900

1194

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03202 - 05 STB

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Computer Simulation of Renal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLI
OTHER:	R. Mejia	Mathematician	OD NHLI
	A. Tsan	Visiting Fellow	OD NHLI
	B. Hubbard	Prof. IFDAM, Univ. of Maryland	
	R. Tewarson	Prof. SUNY, Stony Brook, L.I. New York	
	B. Kellogg	Prof. IFDAM, Univ. of Maryland	

COOPERATING UNITS (if any)

IFDAM, University of Maryland; Department of Applied Mathematics and Statistics, SUNY, Stony Brook, L. I. New York; and NIAMDD, Office of Mathematical Research.

LAB/BRANCH

SECTION
Section on Theoretical Biophysics

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	2.2	.5

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop a computer simulation of the kidney, which describes transport of electrolyte, nonelectrolyte and water in both steady state and transient behavior. Current work is directed toward the development of efficient numerical methods of solving the differential-integral equations describing the renal couterflow system and of estimating model parameters.

1195

Project Description:

Objectives: The purpose of this project is to develop a computer simulation of the mammalian kidney that gives a realistic description of function. This will allow the correlation of micropuncture and macroscopic clearance data with membrane transport characteristics.

Major Findings: (1) A multinephron multisolute model of the whole kidney has been developed. This model includes nephrons, vasculature, and interstitial space, and considers hydrostatic and oncotic pressure, and salt and urea transport. Solutions of this model have been obtained stepwise by solving individual tubes against assumed interstitial concentrations and pressures. The interstitial values are then adjusted using the Newton-Raphson method so as to satisfy mass balance. A multinephron central core medullary model that uses the stepwise solution scheme has also been developed.

These models are being used to model urea cycling and its effect on salt transport in the medulla and to analyze the relation of cortical to juxtamedullary nephron function.

(2) The single nephron model of the whole kidney was extended to include interstitial bulk flow and diffusion, and the effect of protein leak from capillaries. Solutions of this model were obtained by using Tewarson's sparse matrix algorithm. The solutions of this model have been consistent with those obtained with a model that neglects the effects of protein leak into the interstitium. Concentrations and pressure profiles obtained with these whole kidney models are reasonable, but as permeabilities become large, profiles of transmembrane fluxes (which reflect transmembrane driving forces) become irregular and tend to oscillate. This behavior is obviously non-physiological and an artefact of the trapezoidal differencing scheme.

(3) In order to develop computational methods that eliminate the problem of oscillation, a systematic comparison of the accuracy and stability of various algorithms was carried out. An exponential approximation, a backward Euler, an implicit Runge-Kutta, an Adams predictor-corrector, and the centered trapezoidal scheme were used to solve a model of a single tube exchanging water with a bath. The exponential approximation and the implicit Runge-Kutta scheme were found to be most accurate, while the backward Euler had the largest error in mass balance. On the other hand, the Euler scheme and the exponential approximation led to the least oscillation in the solution.

(4) In order to improve the accuracy of the integration, two corrected trapezoidal schemes that use cubic approximations to concentrations have been developed and tested in a six-tube medullary model (Tewarson).

Proposed Course and Biomedical Significance:

Our numerical methods have been developed to the point where it has been possible to formulate and solve a multinephron multisolute model of the kidney that accounts for salt, water, and urea transport and hydrostatic and oncotic pressure. With this model it should be possible to simulate many aspects of renal function.

1196

It is planned to extend the model to include additional features such as a more realistic model of proximal tubule transport and transmembrane potential effects on cation and anion transport.

We also plan to use the model to begin work on the problems of system identification or parameter estimation by correlation of model and kidney input/output data.

Both extension of the model and system identification will require steady development of numerical methods,and continued work in this area, particularly on polynomial approximations, is planned.

Publications:

Stephenson, J. L., Mejia, R., and Tewarson, R.P. Model of solute and water movement in the kidney. Proc. Nat. Acad. Sci. USA 73:252-256,1976.

Tewarson, R. P., Stephenson, J. L., Kydes, A. and Mejia, R. Use of sparse matrix techniques in numerical solution of differential equations for renal counterflow systems. Computers and Biomedical Research (In press).

Tewarson, R. P., and Stephenson, J. L. Solution of large sparse systems of nonlinear equations. College of Engineering, SUNY at Stony Brook, Report No. 265, Nov. 1975.

Stephenson, J. L. Introductory remarks on the solution of kidney models. Conference Proceedings, 1976 Summer Computer Simulation Conference, Washington, D. C. (In press).

Mejia, R., Stephenson, J. L., and Kellogg, R. B. Comparison of numerical methods in solving renal countercurrent equations. Conference Proceedings, 1976 Summer Computer Simulation Conference, Washington, D. C. (In press).

Hubbard, B. E.* Computing transient solutions for certain renal counterflow systems. Conference Proceedings, 1976 Summer Computer Simulation Conference, Washington, D. C. (In press).

*Supported by NHLI under Contract HI 52900

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03203-01 STB

PERIOD COVERED

July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Theory of Epithelial Transport

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. L. Stephenson Chief, Section on Theoretical Biophysics OD NHLI

OTHER: R. Huss NIH Postdoctoral Fellow

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

SECTION

Section on Theoretical Biophysics

INSTITUTE AND LOCATION

NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop the theory of solute and water transport across epithelia. The project is directed particularly toward problems in proximal tubule transport in the mammalian kidney. Present studies center on the effect of changes in hydrostatic, osmotic, and protein oncotic pressure on the osmolality and flow rate of transported fluid in models that include both cellular and shunt pathways.

1198

Project Description:

Objectives: The purpose of this project is to develop the theory of solute and water transport across epithelia. The project is directed particularly at problems in proximal tubule transport in the mammalian kidney. Ultimately we hope to develop models that will account for steady state and time dependent transport of cations, anions, organic solute, and water through cellular and shunt pathways. Current studies center on the effect of changes in hydrostatic, osmotic, and protein oncotic pressure on the osmolality and flow rate of transported fluid.

Methods: Satisfactory results have been obtained in the steady state case using the method of quasilinearization. This method has been found to be superior, for the particular set of equations involved, to shooting methods, since no non-linear differential equations need to be evaluated numerically.

Solution of the models by finite difference methods similar to those used for kidney models is being tested.

Major findings: Previous models of flow through lateral intercellular channels have not considered the cell as a responsive compartment, but rather as a reservoir having known pressure and concentration. These models have now been extended to include flow and concentration profiles in the cell, as well as in the intercellular channels.

Under the conditions that there is no flow (either solute or total mass) across the basal cell membrane into the interstitium, and also that the lateral cell wall is impermeable to solute except by active pumping, the concentration profiles show little variation over the length of the channel (on the order of 2 mOsm). This variation is smaller than that for the case where the cell is considered a reservoir whose pressure and concentration are the same as those of the lumen. This lack of variation of the concentration profile was observed for a wide range of parameter values.

Because of this lack of variation of concentration over the length of the the channel, it was logical to conceive of the system in terms of a compartment model, where both the cell and the channel are well-mixed compartments whose hydrostatic and osmotic pressures are unknown variables of the system.

A compartment model was developed, and the non-linear algebraic equations were solved using the Newton-Raphson method. The resulting numerical values of flows and concentrations in the system agreed very well with those obtained using the differential equation model.

In examining the response of the transmural flow rate to changes in the hydrostatic pressure in the interstitium, it was found that the flow shows a nearly linear response to interstitial pressure, with the tubule lumen pressure remaining constant. The magnitude of the slope is in reasonable agreement with experimental results.

A second variable of interest is the osmolality of the emergent fluid. Since it is established experimentally that transport is virtually isotonic, the model should show this. This was not always the case for all interstitial conditions. If the fluid transported is hypertonic, then the luminal fluid becomes hypotonic. The osmolality of the emergent fluid was found to be highly sensitive to the osmolality of the luminal fluid; if the luminal fluid is hypotonic, the transported fluid is more hypotonic. Thus, the system operates to bring the osmolality of the luminal fluid to a specific value. This value is, however, dependent on the values of the parameters chosen for the rest of the model. Only a preliminary study of parameter values has thus far been accomplished.

In an earlier preliminary study finite difference methods were used to solve the Diamond and Bossert model. The method reproduced the classic solution and seemed very efficient. This suggested applying the method to the more detailed models that include cellular and shunt pathways. A program for solving this model by Newton-Raphson techniques has been developed. Preliminary results indicate that the method is very efficient.

Proposed course: Solution of the parallel path model obtained by quasilinearization and finite difference methods will be compared. A more detailed parameter study will be carried out. It is planned to extend the finite difference method to the time dependent case. A simplified model of proximal tubule transport will be incorporated into the whole kidney model.

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ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1975 through June 30, 1976

Fusion of clonal neuroblastoma cells with rat glioma cells yielded clonal hybrid cell lines which synthesize, store and excrete acetylcholine; properties which are not expressed by the parental cell lines. Cells from one hybrid line were found to form synapses with cultured striated muscle cells. Synapses between hybrid cells and muscle cells closely resemble the synapses between normal motor neurons and striated muscle before they are fully developed. Under appropriate conditions, hybrid cells establish synaptic connections with virtually every muscle cell tested; thus, synaptic connections are formed in abundance. Marked differences were observed in the efficiency of transmission across different synapses. Axonal activities which were found to be regulated include choline acetyltransferase, acetylcholinesterase, Na^+ action potential ionophore specific activities, and the rate of choline transport into cells.

Eight species of receptors have been found thus far with the hybrid cell line which forms synapses. Receptor mediated shifts in cAMP levels, cGMP levels and membrane potentials have been identified and characterized. Thus, the foundation has been laid for studies on the effects of receptor-mediated reactions on synaptic transmission. In addition, more than 100 cell lines which synthesize acetylcholine have been obtained and are being studied to determine whether some cell lines are defective with respect to synapse formation. Much remains to be done but it seems clear that the experimental approach and model systems which have been established afford extraordinary opportunities to explore synapse properties and correlate biochemical events with developmental and electrophysiological phenomena.

Synaptogenesis by normal neurons also was studied. Neurons dissociated from chick embryo retina and maintained *in vitro* were found to reaggregate and form *in vitro* approximately 1×10^9 synapses per mg of protein. Three types of synapses and several subtypes were identified which closely resemble those of the intact retina. Studies with this system are described in other sections of this report.

A histochemical technique for detecting and localizing nicotinic acetylcholine receptors was devised previously which depends upon the formation of a complex between peroxidase coupled to an antibody for α-bungarotoxin and the nicotinic acetylcholine receptor. Using this method, clusters of nicotinic acetylcholine receptors on cultured muscle cells were shown to contain at least 7 times the concentration of receptors found in other membrane regions. Receptor clusters are not characteristically associated with folds in the plasma membrane.

The hybrid cells which form synapses possess abundant morphine receptors. Morphine and other narcotics were shown to be potent inhibitors of adenylate cyclase in cells which possess opiate receptors but not in cells which lack these receptors. Exposure of cells with opiate receptors to morphine for 12 to 48 hours results in an increase in adenylate cyclase activity which compensates for the inhibition of enzyme activity by morphine. Cells have normal

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cAMP levels and appear tolerant to morphine because the increase in adenylate cyclase activity is approximately equal to the inhibition of enzyme activity by morphine. However, the cells then are dependent upon morphine to maintain normal cAMP levels. Withdrawal of morphine, or displacement of the narcotic from the opiate receptor by the antagonist, naloxone, reverses the inhibition and results in the synthesis of abnormally high levels of cAMP. Thus, dual regulation of adenylate cyclase by narcotics accounts for the phenomena of narcotic dependence and tolerance. The recently discovered endogenous opiate peptides, Met-enkephalin and Leu-enkephalin, also were shown to be potent inhibitors of adenylate cyclase. These results show that the endogenous opiate peptides and narcotics act as pleiotropic regulators of other species of receptors which are coupled to the activation of adenylate cyclase. By this mechanism opiates alter the perception of neurons to incoming messages.

A cell line with muscarinic inhibitory acetylcholine receptors, and another line with muscarinic excitatory receptors were found. The number of receptors, the receptor affinities for cholinergic ligands and other receptor properties were determined by measuring the binding of [^{3}H]-quinuclidinyl benzilate to receptors. The apparent dissociation constant of the ligand·receptor complex is 6×10^{-11} M. Activation of muscarinic acetylcholine receptors results in a transient increase in cGMP, and a profound, long-lived inhibition of adenylate cyclase activity. Exposure of cells to acetylcholine or carbachol for 24 hours markedly decreases the number of acetylcholine receptors and increases adenylate cyclase activity 50 to 500%. Removal of carbachol results in the return of adenylate cyclase activity and acetylcholine receptor levels to normal values after approximately 3 and 24 hours, respectively. Similarly, exposure of cells with α-receptors to norepinephrine inhibits adenylate cyclase activity and elicits a delayed, compensatory increase in adenylate cyclase activity. These results show that prolonged activation of α-receptors, muscarinic acetylcholine receptors, or opiate receptors results in cell tolerance to and dependence upon norepinephrine, acetylcholine, or opiates, respectively. Withdrawal of the receptor activator elevates intracellular cAMP levels and shifts cells to a supersensitive state with respect to other species of receptors which activate adenylate cyclase.

Desensitization of muscarinic receptors decreases the affinity of the receptor for agonists by a process which exhibits negative cooperativity; whereas interactions between the receptor and antagonists are not cooperative. Both muscarinic excitatory and inhibitory acetylcholine receptors were solubilized and the properties of membrane-bound and soluble receptors were compared.

Chick embryo retina was found to be a rich source of both muscarinic and nicotinic acetylcholine receptors. Both muscarinic and nicotinic acetylcholine receptors are synthesized before synapses appear in the retina; however, during development, nicotinic acetylcholine receptors become associated predominantly with neurites in the synaptic layers of the retina. The properties of muscarinic acetylcholine receptors were determined at different developmental ages and were compared with the properties of muscarinic inhibitory and excitatory receptors of neuroblastoma cells.

Evidence for a new type of PGE_1 receptor coupled to cGMP accumulation was obtained. Cell lines with PGE_1 receptors coupled only to cAMP were found as well as cell lines with 2 species of PGE_1 receptors, one coupled to cAMP accumulation, the other to cGMP accumulation. The 2 species of PGE_1 receptors also desensitize at different rates. These results show that the coupling of PGE_1 to increases in cAMP and cGMP levels are clonally inherited properties which can be expressed independently.

Two pathways for γ-aminobutyric acid synthesis were found in chick embryo retina. The first pathway depends upon the conversion of putrescine to ornithine, catalyzed by ornithine decarboxylase and the subsequent conversion of ornithine to γ-aminobutyric acid. The second route of synthesis is dependent upon the conversion of glutamic acid to γ-aminobutyric acid, catalyzed by glutamic acid decarboxylase. Elevation of cAMP levels in neuroblastoma cells was shown to induce ornithine decarboxylase activity. In the developing embryo, neurotransmitters which affect cAMP levels may regulate ornithine decarboxylase activity and thereby control the rate of GABA synthesis from ornithine.

Previous results led to the conclusions that (1) veratridine, batrachotoxin, and aconitine activate the action potential Na^+ ionophore by interaction with a single class of sites; (2) scorpion venom activates the ionophore by interaction with a different class of sites; (3) two species of toxin bound to separate sites are allosterically coupled and interact in a cooperative manner; and (4) tetrodotoxin and saxitoxin act at a 3rd site which is involved in ion transport.

A toxin which activates the action potential Na^+ ionophore has been purified from scorpion venom. The toxin binds to a single class of sites and acts cooperatively with each of the three alkaloids. Depolarization of cells causes a 30-fold increase in the apparent dissociation constant. The results suggest that the scorpion toxin binds to a voltage sensitive component of the Na^+ ionophore that acts cooperatively in regulating ion transport activity. Binding studies with an ^{125}I-labelled derivative of scorpion toxin showed that the concentration of toxin binding sites is approximately 3 to 6 fmole per mg protein.

Clonal skeletal muscle myoblasts have substantial action potential Na^+ ionophore activity. A small increase in activity accompanies cell fusion. The activity in both myoblasts and myotubes is relatively insensitive to inhibition by saxitoxin and tetrodotoxin and thus resembles denervated rat striated muscle which has been shown to be relatively insensitive to these toxins. Chronic electrical stimulation of muscle cells <u>in vitro</u> does not increase tetrodotoxin sensitivity.

At least 3 ionophores are involved in the action potential in adult heart: a rapidly activated axon-like Na^+ ionophore responsible for the rising phase of the action potential, a slower Ca^{++}/Na^+ ionophore responsible for the plateau phase, and a K^+ ionophore responsible for the repolarization phase. Studies with specific inhibitors of the fast Na^+ ionophore (tetrodotoxin) and the slow Ca^{++}/Na^+ ionophore (D-600) show that the role of these two types of ionophore in beating changes during development of the embryonic chick heart.

In early embryonic hearts, the fast Na^+ ionophore is present but is not required for beating. During development *in ovo* or in monolayer or aggregate culture *in vitro*, changes in the requirement for activity of the fast Na^+ ionophore in beating are accompanied by changes in the sensitivity of the slow Na^+/Ca^{++} ionophore to D-600. When the slow Na^+/Ca^{++} ionophore is able to maintain beating without participation of the fast Na^+ ionophore, its sensitivity to D-600 is high whereas when the fast Na^+ ionophore is required for beating, the slow Na^+/Ca^{++} ionophore is relatively insensitive to D-600. Transitions between these two states can be induced in 2 hours *in vitro* by inhibition of beating. Thus, the activity of these ionophores is regulated during development by a process dependent on the rhythmic activity of the cells.

The developmental changes in action potential ionophores of embryonic hearts occur between days 4 and 7 *in ovo*. During this time, vagal innervation of the heart takes place. Consequently we have studied the muscarinic acetylcholine receptors in embryonic hearts using both physiologic and ligand binding methods to determine whether changes in their properties are temporally correlated with changes in the action potential ionophores. In early embryonic hearts, muscarinic agents are ineffective in inhibiting beating. Between days 5 and 7, sensitivity to inhibition by muscarinic agents increases to the adult level. QNB binds specifically to muscarinic acetylcholine receptors in embryonic and adult heart as assessed by competition studies with muscarinic and nicotinic agents. Receptors, as detected by QNB binding, are present in unresponsive early embryonic hearts and the number per mg heart protein does not increase dramatically during development. Receptor desensitization is accompanied by a small change (3 fold increase) in the K_D for muscarinic agonists as measured by competition with ^3H QNB. This change in K_D occurs only in hearts that are responsive to muscarinic agents and thus may be associated with the physiologic action of the receptor. Competition curves for agonists suggest the involvement of negative cooperativity in activation of the receptor. These results suggest that muscarinic acetylcholine receptors, like action potential ionophores, are present in early embryonic hearts in modified "precursor" or "inactive" forms and undergo activation during development.

Studies on the mechanism of catabolite repression in *E. coli* show that glucose inhibits adenylate cyclase activity reversibly in cells treated with toluene, and that phosphate is required for high adenylate cyclase activity and for glucose dependent inhibition of enzyme activity. Other sugars also inhibit adenylate cyclase provided that transport systems for the sugars are induced. Mutant strains of *E. coli* defective in phosphoenolpyruvate:sugar phosphotransferase system components were examined. Cyclic AMP levels were normal in an HPr mutant, but were markedly depressed in a leaky Enzyme I mutant. Adenylate cyclase activity was low in the Enzyme I mutant whereas the HPr mutant had normal enzyme activity. The Enzyme I mutant under starvation conditions exhibits high adenylate cyclase activity and the adenylate cyclase of this mutant is unusually sensitive to variations in carbon source. The addition of phosphoenolpyruvate leads to a substantial increase in adenylate cyclase activity in permeabilized cell preparations of the Enzyme I mutant. These results suggest that Enzyme I is involved in the regulation of adenylate cyclase activity. Studies are in progress to test the hypothesis that Enzyme I interacts with adenylate cyclase and that the PEP-dependent phosphorylation of Enzyme I is responsible for activation of adenylate cyclase.

Levels of cGMP were compared with cAMP levels in E. coli grown under different conditions. The results show that cGMP and cAMP concentrations are inversely coupled in E. coli and that the regulation of cGMP levels can be uncoupled from that of cAMP.

Since specific species of tRNA are involved in amino acid mediated repression and end-product inhibition, the effect of amino acid deprivation upon tRNA of relaxed and stringent strains of E. coli was studied. In relaxed control E. coli, leucine starvation results in the formation of new isoacceptor species of leucine, histidine, arginine, valine, and alanine-specific tRNA and quantitative changes in the concentration of some other isoacceptors. Experiments with stringent strains or the use of uracil starvation or rifampicin addition provided evidence for the de novo synthesis of new species of leucine-tRNA. The new species of leucine-tRNA is not formed by aggregation of tRNA or nuclease catalyzed hydrolysis, and it is not grossly deficient with respect to methylation. Since there is some evidence from the recent work of others that tRNA formed under conditions of amino acid starvation is deficient in the minor bases 5,6-dihydrouridine and 4-thiouridine, we think that the biochemical explanation for the accumulation of new tRNA species under conditions of amino-acid starvation may be that the enzymes responsible for these tRNA modifications are unstable and require continued protein synthesis to maintain their levels of activity.

Further information was obtained on the mechanism of arginyl-tRNA synthetase which does not catalyze an amino acid dependent ATP-PPi exchange in the absence of added tRNA. A new purification procedure was devised which yields homogeneous enzyme in approximately 10% yield. Pulse labelling experiments indicate that no enzyme bound arginyl-adenylate is formed in the absence of added tRNA. Equilibrium experiments show that no arginyl-adenylate accumulates either in the presence or absence of $tRNA^{arg}$. These data further validate our earlier suggestion that the mechanism of this reaction is probably concerted.

A series of additional studies carried out with the purified preparation of arginyl-tRNA synthetase from E. coli indicate that metals may have two functional roles in the catalytic mechanism. Complete metal activation is observed when $MgCl_2$, $MnCl_2$, $CoCl_2$, or $FeCl_2$ is present at a concentration (5 mM) in excess of the total ATP concentration (2 mM). When $CaCl_2$ is substituted for $MgCl_2$, activity is not observed unless a small amount (0.1 mM) of $MgCl_2$, $MnCl_2$, $CoCl_2$, $FeCl_2$ or $ZnCl_2$ is added. On the basis of these experiments, we visualize a model in which the enzyme possesses a site for free metal which, when filled, lowers the K_m for the three substances (arginine, $tRNA^{arg}$, and metal·ATP) and increases the V_{max} of the reaction.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00001-05 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mathew P. Daniels	Staff Fellow	LBG NHLI
OTHER:	Marshall W. Nirenberg	Chief, Lab. of Biochem. Genetics	LBG NHLI
	P. Nelson	Chief, Behavioral Biology Branch	LBG NHLI
	C. Christian	Special Fellow	BB NICHD
	G. Maloney	NIH Postdoctoral Fellow	LBG NHLI
	Zvi Vogel	Assistant Professor	Weizmann Institute

COOPERATING UNITS (if any)
Behavioral Biology Branch, NICHD
Neurobiology Unit, Weizmann Institute of Science

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

SUMMARY OF WORK (200 words or less - underline keywords)

Our aim is to study the distribution of nicotinic acetylcholine receptors in intact and cultured tissues of the peripheral and central nervous system in relationship to the development and function of synapses. To this purpose histochemical localization of α-bungarotoxin bound to the receptors is used in conjunction with light and electron microscopy. In the past year we have studied the ultrastructural distribution of receptors on cultured skeletal muscle fibers and have initiated the following investigations: 1) location and characterization of synapses formed by neuroblastoma hybrid cells in culture 2) modification of histochemical methods in order to permit ultrastructural analysis of receptor distribution in the central nervous system and 3) analysis of the distribution of receptors in the visual system of the goldfish with relationship to optic nerve damage and regeneration.

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Project Description:

Objectives: Investigators in this laboratory and others have utilized ^{125}I labelled α-bungarotoxin (αBT) as a label for nicotinic acetylcholine receptors in intact and cultured skeletal muscle, and in embryonic and mature retina. The objectives of this study were to devise a histochemical technique of greater sensitivity and resolution for localizing bound αBT and to apply this technique to studying the ultrastructural distribution of acetylcholine receptors in the peripheral and central nervous system during development, in culture, and in the mature state.

Methods Employed: We have employed indirect immunoperoxidase staining of cryostat sectioned, teased, or monolayered cultured materials to which αBT has been bound. These materials are subsequently examined by light and electron microscopy.

Major Findings: Acetylcholine receptor-rich regions on the surface of muscle fibers grown in culture had previously been observed by light microscope autoradiography with [^{125}I]-labelled αBT. The appearance of these regions could be explained either by (1) the localized presence of complex folds in the plasma membrane or (2) a high local concentration of receptors in the plasma membrane, unrelated to membrane folding. Using the αBT-immunoperoxidase technique with light- and electronmicroscopy we have shown that hypothesis 2 is correct; the plasma membranes of these regions contain at least 7 times the concentration of receptors found in other regions, with no distinctions in cell surface topography.

Significance to Biomedical Research: Knowledge of ultrastructural distribution of acetylcholine receptor is of clear importance in any attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system. The α-bungarotoxin-immunoperoxidase technique already has shown promise for the diagnosis and analysis of mechanisms in human neuromuscular disorders.

Proposed Course: (1) We are using the αBT-immunoperoxidase technique to help locate and characterize the ultrastructure of synapses which have been detected electrophysiologically in cultures of neuroblastoma hybrid cells with skeletal muscle fibers. (2) We are developing new reagents to adapt the histochemical technique to ultrastrucutral visualization of acetylcholine receptor sites in mature and developing central nervous system tissues. (3) We plan to study the distribution of acetylcholine receptors in the visual system of the goldfish with relationship to the destruction and reformation of synapses during optic nerve degeneration and regeneration.

Publications:

1. Ringel, S. P., Bender, A. N., Festoff, B. W., Engel, W. K., Vogel, Z. and Daniels, M. P.: Ultrastructural demonstration and analytical application of extrajunctional receptors of denervated human and rat skeletal muscle fibres. Nature 255: 730-731, 1975.

2. Vogel, Z. and Daniels, M. P.: The ultrastructure of acetylcholine receptor clusters on cultured muscle fibers. J. Cell Biol. 69: in press.

3. Vogel, Z. and Daniels, M. P.: The acetylcholine receptor of intact and cultured chicken retina cells. Proc. VI Int. Cong. Pharmacol. Vol. 1 Receptors and Cellular Pharmacology. Pergamon Press, New York, 1976, pp. 59-66.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00002-03 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Morphine Receptors as Regulators of Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, Lab. of Biochemical Genetics	LBG NHLI
OTHER:	Arthur Lampert	Staff Fellow	LBG NHLI
	Werner Klee	Research Chemist	LGCB NIMH

COOPERATING UNITS (if any)

Laboratory of General and Comparative Biochemistry, NIMH

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives are to elucidate the mechanisms of dependence upon opiates and of tolerance to these compounds and to define the normal functions of the newly discovered endogenous opiate peptides.

1210

Project Description:

Major Findings: Clonal cell lines with morphine receptors were found and were used to study the mechanism of action of narcotics. Morphine and other narcotics were found to affect adenylate cyclase in two ways, mediated by the opiate receptor: (1) narcotics inhibit adenylate cyclase activity, and (2) when cells are cultured in the presence of morphine for 12 to 48 hours an increase in adenylate cyclase activity is observed which compensates for the inhibition of enzyme activity by morphine. Cells then have normal cAMP levels and appear tolerant to morphine because the increase in adenylate cyclase activity is approximately equal to the inhibition of enzyme activity by morphine. However, the cells then are dependent upon morphine to maintain normal cAMP levels. Withdrawal of morphine, or displacement of the narcotic from the opiate receptor by the antagonist, naloxone, reverses the inhibition and results in the synthesis of abnormally high levels of cAMP. Thus, dual regulation of adenylate cyclase by narcotics accounts for narcotic dependence and tolerance. The recently discovered endogenous opiate peptides, Met-enkephalin and Leu-enkephalin, also were shown to be potent inhibitors of adenylate cyclase. These results show that the endogenous opiate peptides and narcotics act as pleiotropic regulators of other species of receptors which are coupled to the activation of adenylate cyclase. In this way, the opiates alter the perception of neurons to incoming messages.

Significance to Biomedical Research: The biochemical basis for narcotic dependence and tolerance has been established and the mode of action and the normal role of the endogenous opiate peptides have been clarified.

Proposed Course: Further studies on the mechanism of dual regulation of adenylate cyclase are in progress.

Publications:

1. Sharma, Shail K., Klee, Werner A. and Nirenberg, Marshall: Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc. Natl. Acad. Sci. USA 72: 3092-3096, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00003-03 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of Receptor Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, Lab. of Biochemical Genetics	LBG NHLI
	Hiroshi Matsuzawa	Visiting Associate	LBG NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1	.3

SUMMARY OF WORK (200 words or less - underline keywords)

Evidence for a new type of PGE_1 receptor coupled to cGMP accumulation was obtained. Cell lines with PGE_1 receptors coupled only to cAMP were found as well as cell lines with 2 species of PGE_1 receptors, one coupled to cAMP accumulation, the other to cGMP accumulation. The 2 species of PGE_1 receptors also desensitize at different rates. These results show that the coupling of PGE_1 to increases in cAMP and cGMP levels are clonally inherited properties which can be expressed independently.

1212

PHS-6040
(12-75)

Project Description:

Objectives: The objective is to define receptor-mediated responses of clonal cells which can be used as model systems for synapse studies.

Major Findings: Evidence for a new type of PGE_1 receptor coupled to cGMP accumulation was obtained. Cell lines with PGE_1 receptors coupled only to cAMP were found as cell lines with 2 species of PGE_1 receptors, one coupled to cAMP accumulation, the other to cGMP accumulation. The 2 species of PGE_1 receptors also desensitize at different rates. These results show that the coupling of PGE_1 increases in cAMP and cGMP levels are clonally inherited properties which can be expressed independently.

Publications:

1. Matsuzawa, Hiroshi and Nirenberg, Marshall: Receptor-meeiated shifts in cGMP and cAMP levels in neuroblastoma cells. Proc. Natl. Acad. Sci. USA 72: 3472-3476, 1975.

2. Bachrach, Uriel: Cyclic AMP-mediated induction of ornithine decarboxylase of glioma and neuroblastoma cells. Proc. Natl. Acad. Sci. USA 72: 3087-3091, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00004-03 LBG

PERIOD COVERED
July 1, 1975 to June 30, 1976

TITLE OF PROJECT (80 characters or less)

Studies of Action Potential and Receptor Ionophores

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. A. Catterall	Staff Fellow	LBG NHLI
	R. Ray	MARC Fellow/NIGMS	LBG NHLI
	L. M. Huang	NIH Postdoctoral Fellow	LBG NHLI and BP NINCDS

COOPERATING UNITS (if any)

Laboratory of Biophysics, NINCDS

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.7	1

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to develop biochemical methods for studies of action potential and receptor ionophores leading eventually to isolation of these macromolecules and characterization at both the molecular and cellular levels. Our current efforts are directed toward developing reagents, mainly neurotoxins, which act on the action potential Na^+ ionophore, preparing radioactively labelled derivatives, and using these reagents to characterize the ionophore at the cellular level and to solubilize and eventually isolate it. The nicotinic acetylcholine receptor ionophore is also under study at the cellular level.

1214

Project Description:

Objectives: The objectives of this project are (1) to develop biochemical methods for study of action potential and receptor ionophores, (2) to use these methods to study the mechanism of action of these macromolecules at the cellular and membrane levels, and (3) to solubilize, purify, and characterize these ionophores at the molecular level.

Methods Employed: Biochemical assays which measure changes in passive Na^+ influx were used to study the acetylcholine receptor ionophore and the action potential Na^+ ionophore.

Major Findings: Previous results led to the conclusion that (1) the neurotoxic alkaloids veratridine, batrachotoxin, and aconitine activate the action potential Na^+ ionophore by interaction with a single class of sites; (2) scorpion venom activates the ionophore by interaction with a different class of sites; (3) the sites of action of these 2 classes of toxin are allosterically coupled in a highly cooperative manner; and (4) the inhibitors tetrodotoxin and saxitoxin act at a separate site directly involved in ion transport by the ionophore.

The active component of scorpion venom has been purified using its ability to activate the action potential Na^+ ionophore as a specific assay. The toxin is a polypeptide having a molecular weight of 6700, an isoelectric point of 9.8, and lacking methionine and histidine. The purified toxin retains the ability to act cooperatively with each of the 3 alkaloids. It acts reversibly at a single class of sites with an apparent dissociation constant of 1 to 2 nM. The action of the toxin is highly membrane potential dependent. Depolarization of the cells causes a 30 fold increase in apparent dissociation constant. These results suggest that scorpion toxin binds to a voltage sensitive component of the Na^+ ionophore that acts cooperatively in regulating its ion transport activity.

We have prepared an ^{125}I-labelled derivative of scorpion toxin which retains biologic activity. Using this derivative we have detected a small class of saturable binding sites in electrically excitable neuroblastoma cells but not in neuroblastoma cells defective in electrical activity. Binding of scorpion toxin to these sites is voltage dependent as is the effect of the toxin on ion transport activity. Preliminary estimates of the number of sites are in the range of 3 to 6 fmole/mg cell protein or less than 1 site per μm^2 of cell membrane. This labelled toxin derivative appears to provide an important new tool in studies of the Na^+ ionophore.

Significance to Biomedical Research: The results provide new insights into the mechanism of action and regulation of membrane macromolecules involved in information transfer and processing in the nervous system and in maintenance of normal beating in heart.

Proposed Course: Planned investigations include (1) completing the analysis of scorpion toxin binding to excitable membranes of neuroblastoma cells, nerve axons, and heart muscle; (2) preparing labelled derivatives of saxitoxin and comparing binding with scorpion toxin; (3) studying the voltage dependent aspects of scorpion toxin binding in detail and relating them to the electrophysiologic properties of the ionophore; and (4) attempting to solubilize and purify the binding sites for scorpion toxin and saxitoxin and thus isolate the action potential Na^+ ionophore.

Publications:

1. Catterall, W. A.: Activation of the action potential Na^+ ionophore of cultured neuroblastoma cells by veratridine and batrachotoxin. J. Biol. Chem. 250: 4053-4059, 1975.

2. Catterall, W. A.: Cooperative activation of the action potential Na^+ ionophore by neurotoxins. Proc. Natl. Acad. Sci. USA 72: 1782-1786, 1975.

3. Catterall, W. A. and Ray, R.: Interactions of neurotoxins with the action potential Na^+ ionophore. J. Supramolecular Structure, in press.

4. Catterall, W. A.: Purification of a toxic protein from scorpion venom which activates the action potential Na^+ ionophore. J. Biol. Chem., in press.

5. Catterall, W. A., Ray, R. and Morrow, Cynthia S.: Membrane potential dependent binding of scorpion toxin to the action potential Na^+ ionophore. Proc. Natl. Acad. Sci. USA, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00009-02 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cell Recognition and Synapse Formation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, Lab. of Biochemical Genetics	LBG NHLI
OTHER:	Donald Puro	Research Associate	LBG NHLI
	Hiroyuki Sugiyama	Visiting Associate	LBG NHLI
	Phillip Nelson	Chief, Behavioral Biology Branch	BB NICHD
	Clifford Christian	Special Fellow	BB NICHD

COOPERATING UNITS (if any)

Behavioral Biology Branch, NICHD

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	2	.2

SUMMARY OF WORK (200 words or less - underline keywords)

Long range objectives are to define reactions which are required for synapse formation and for transmission of information from cell to cell.

1217

PHS-6040
(12-75)

Project Description:

Major Findings: Since normal neurons do not divide, clonal lines of neuroblastoma cells and somatic cell hybrids derived from neuroblastoma cells were generated and characterized with respect to receptors, neurotransmitters, action potential ionophores, and other properties which are required for synaptic communication. Fusion of clonal neuroblastoma cells with glioma cells yielded clonal hybrid cell lines which synthesize, store and excrete acetylcholine; properties which are not expressed by the parental cell lines. During the past year cells from one hybrid line were found to form synapses with cultured striated muscle cells. Synapses between hybrid cells and muscle cells closely resemble the synapses between normal motor neurons and striated muscle before they are fully developed. Under appropriate conditions, hybrid cells established synaptic connections with almost every muscle cell tested; thus, synapses are synthesized in abundance. Marked differences were observed in the efficiency of transmission across different synapses. Axonal activities which were found to be regulated include choline acetyltransferase, acetylcholinesterase, Na^+ action potential ionophore specific activities, and the rate of choline transport into cells.

Eight species of receptors have been found thus far with the hybrid cell line which forms synapses. Receptor mediated shifts in cAMP levels, cGMP levels and membrane potentials have been identified and characterized. Thus the foundation has been laid for studies on the effects of receptor-mediated reactions on synaptic transmission. In addition, more than 100 cell lines which synthesize acetylcholine have been obtained and are being studied to determine that some cell lines are defective with respect to synapse formation.

Significance to Biomedical Research: This is the first time that clonal cells of neural origin have been shown to form synapses. Thus the set of genes which are required for synapse formation are expressed in this cell line. The model system which has been established affords extraordinary opportunities to define synapse formation reactions and properties and correlate biochemical events with developmental and electrophysiological phenomena.

Proposed Course: Current studies focus on determining the reactions which are required for synapse formation and factors that regulate these reactions.

Publications:

1. Greene, Lloyd A., Shain, William, Chalazonitis, Alcmene, Breakefield, Xandra, Minna, John, Coon, Hayden G. and Nirenberg, Marshall: Neuronal properties of hybrid neuroblastoma x sympathetic ganglion cells. Proc. Natl. Acad. Sci. USA 72: 4923-4927, 1975.

2. Chalazonitis, A., Greene, L.A. and Shain W.: Excitability and chemosensitivity properties of a somatic cell hybrid between mouse neuroblastoma and sympathetic ganglion cells. Exp. Cell Res. 96: 225-238,1975.

3. Nelson, Phillip, Christian, Clifford and Nirenberg, Marshall: Synapse formation between clonal neuroblastoma x glioma hybrid cells and striated muscle cells. Proc. Natl. Acad. Sci. USA 73: 123-127, 1976.

4. Nirenberg, Marshall W.: Coding of neural information by neuroblastoma cells. In: Talwar, G. P. (Ed.): Regulation of Growth and Differentiated Function in Eukaryote Cells. New York, Raven Press, pp. 537-539, 1975

5. Thompson, Edward J., Griffith, James M., Glazer-Schoenberg, Deverera and Nirenberg, Marshall W.: An improved method for extracellular recording of action potentials from single cultured neuroblastoma cells. Med. Biol. Eng. 13: 104-106, 1975.

6. Hamprecht, Bernd, Kemper, Wayne and Amano, Takehiko: Electrical response of glioma cells to acetylcholine. Brain Res. 101: 129-135, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00011-02 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976.

TITLE OF PROJECT (80 characters or less)

The Development of Chick Embryo Retina

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. G. DeMello	Visiting Associate	LBG NHLI
	Marshall Nirenberg	Chief, LBG	LBG NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

SUMMARY OF WORK (200 words or less - underline keywords)

Some biochemical aspects of chick embryo retina differentiation were studied: 1) An alternate route for GABA synthesis was characterized in the retina; which depends upon the conversion of putrescine to GABA. 2) Glutamic acid decarboxylase specific activity in the retina, increased during the course of embryonic development, either when measured in ovo or in aggregate cultures. 3) The presence of GABA in the culture medium prevented the development of glutamic acid decarboxylase activity in aggregate cultures. 4) The proposed course of this project is to attempt to correlate the biochemical changes observed, with synaptogenesis in the retina.

1220

PHS-6040
(12-75)

Project Description:

Objectives: The objective of the project is to study the biochemical step required for synaptogenesis in chick embryo retina.

Major Findings: Two pathways for γ-aminobutyric acid synthesis were found in chick embryo retina. The first pathway depends upon the conversion of putrescine to ornithine decarboxylase and the subsequent conversion of ornithing to γ-aminobutyric acid. The second route of synthesis is dependent upon the conversion of glutamic acid to γ-aminobutyric acid, catalyzed by glutamic acid decarboxylase. Elevation of cAMP levels in neuroblastoma cells was shown to induce ornithine decarboxylase activity. Thus, in the developing embryo, neurotransmitters which affect cAMP levels may regulate ornithine decarboxylase activity and thereby control the rate of GABA synthesis from ornithine.

Proposed Course: To determine the effect of retina neurotransmitters and other compounds on retina synaptogenesis.

Publications:

1. DeMello, F. G., Bachrach, U. and Nirenberg, M.: Ornithine and glutamic acid decarboxylase activities in the developing retina. J. Neurochem. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00012-02 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Muscarinic Acetylcholine Receptors of Cultured Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, Lab. of Biochemical Genetics	LBG NHLI
OTHER:	William Klein	Postdoctoral Fellow	LBG NHLI
	Orest Hurko	Staff Fellow	LBG NHLI
	Wolfgang Burgermeister	Guest Worker	LC NIAMDD
	Bernhard Witkop	Chief, Laboratory of Chemistry	LC NIAMDD

COOPERATING UNITS (if any)

Laboratory of Chemistry, NIAMDD

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

SUMMARY OF WORK (200 words or less - underline keywords)

Our work on muscarinic acetylcholine receptors focuses on two fundamental questions:

(1) How is binding of acetylcholine to receptors converted into an intracellular response?

(2) What factors regulate receptor concentrations in the membrane?

A binding assay employing tritiated quinuclidinyl benzilate permits us to investigate properties of receptor binding sites and to measure receptor levels in cultured neuroblastoma and hybrid cells under systematically varied conditions.

1222

PHS-6040
(12-75)

Project Description:

Objectives: The objectives of our work have been (1) to characterize the nature of ligand interactions with muscarinic receptors, and (2) to assess various factors likely to affect receptor concentrations. Although most methodology involved has been straightforward, our experiments required the custom labeling and purification of muscarinic ligands as commercially available materials were unsuitable due to low specific activity.

Major Findings: The principal findings are:

(1) Excitatory and inhibitory muscarinic receptors for ligands which do not differ pharmacologically.

(2) The affinity of receptors for acetylcholine is affected by the ionic composition of the medium.

(3) Antagonists and agonists differ strikingly in the way that they interact with the receptor. Antagonist binding to receptor follows the law of mass action whereas agonist binding does not. When analyzed on Hill plots, agonist interactions with receptor exhibit negative cooperativity that may be related to receptor desensitization.

(4) The receptor concentration is greatly reduced by exposure of cultured cells to carbamylcholine or other receptor activators. The [receptor·agonist] complex is degraded more rapidly than the free receptor.

(5) Muscarinic excitatory and inhibitory acetylcholine receptors have been solubilized and have been characterized partially.

Significance to Biomedical Research: This work with a model system suggests that, in the normal nervous system, receptor levels, which ultimately influence the pattern of behavior of neural nets, are regulated by synaptic transmission. Some of the events which result from the interaction of acetylcholine with muscarinic acetylcholine receptors have been defined. Two regulatory processes were found which depend upon acetylcholine. One regulates receptor activity, the other regulates receptor concentration.

Proposed Course: Further elucidation of QNB binding will be pursued.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00013-02 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Developmental Regulation of Excitability

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William A. Catterall, Staff Fellow, LBG NHLI
Jonas B. Galper, Clinical Associate, LBG NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to define the role of receptors and action potential ionophores in the development of excitable tissue. Our current efforts are directed toward understanding the role of electrical stimuli, cell-cell interaction, and nerve-muscle interaction in the developmental changes of action potential Na^+ and Ca^{++} ionophores in heart and skeletal muscle.

1224

PHS-6040
(12-75)

Project Description:

Objectives: The objectives of this project are (1) to devise biochemically manipulable cell culture systems for studying the development of action potential generation and receptor function in excitable cells, (2) to use these cell culture systems to document the changes in action potential generation and receptor function occurring during development, and (3) to understand the role of hormonal stimuli, cell-cell interaction, and synapse formation in these developmental changes.

Methods Employed: Cultures of clonal lines rat skeletal muscle and cultures of primary embryonic chick muscle were prepared by standard procedures. Primary cultures of chick embryomic heart cells were prepared either as monolayers of individual beating cells or as suspension cultures of aggregates of a few hundred synchronously beating cells. The ion transport activity of action potential and receptor ionophores was studied using measurements of ^{22}Na influx as described previously (Project NHLI-307). Muscarinic acetylcholine receptors were detected by binding of ^{3}H-quinuclidinyl benzilate (QNB) prepared as described in another project report (Project No. Z01 HL 00012-02 LBG).

Major Findings: (1) Clonal lines of rat skeletal muscle cells cultured as myoblasts have substantial action potential Na^+ ionophore activity. A small increase in activity occurs concomitant with fusion into multinucleate myotubes *in vitro*. Thus, this differential property is expressed in undifferentiated myoblasts. The activity in both myoblasts and myotubes is relatively insensitive to inhibition by saxitoxin and tetrodotoxin (K_I's of 1×10^{-7} and 1×10^{-6} M respectively). Rat skeletal muscle denervated *in vitro* also becomes insensitive to these toxins. Thus, cultured muscle behaves like muscle denervated *in vivo*. Some consequences of denervation can be reversed by electrical stimulation of the muscle *in vivo*. We have thus far been unable to induce tetrodotoxin sensitivity by chronic electrical stimulation of muscle cells *in vitro*.

(2) At least 3 distinct ionophores are involved in the action potential in adult heart: a rapidly activated axon-like Na^+ ionophore responsible for the rising phase of the action potential, a slower Ca^{++}/Na^+ ionophore responsible for the plateau phase, and a K^+ ionophore responsible for the repolarization phase. Studies with specific inhibitors of the fast Na^+ ionophore (tetrodotoxin) and the slow Ca^{++}/Na^+ ionophore (D-600) show that the role of these two types of ionophore in beating changes during development of the embryonic chick heart. In early embryonic hearts, the fast Na^+ ionophore is present but is not required for beating. During development *in ovo* or in monolayer or aggregate culture *in vitro*, changes in the requirement for activity of the fast Na^+ ionophore in beating are accompanied by changes in the sensitivity of the slow Na^+/Ca^{++} ionophore to D600. When the slow Na^+/Ca^{++} ionophore is able to maintain beating without participation of the fast Na^+ ionophore, its sensitivity to D-600 is high whereas when the fast Na+ ionophore is required for beating, the slow Na^+/Ca^{++} ionophore is relatively insensitive to D-600. Transitions between these two states can be induced in 2 hours *in vitro* by beating. Thus, the activity of these ionophores is regulated during development by a process dependent on the rhythmic activity of the cells.

(3) The developmental changes in action potential ionophores of embryonic hearts occur between days 4 and 7 in ovo. During this time, vagal innervation of the heart takes place. Consequently we have studied the muscarinic acetylcholine receptors in embryonic hearts using both physiologic and ligand binding methods to determine whether changes in their properties are temporarily correlated with changes in the action potential ionophores. In early embryonic hearts, muscarinic agents are ineffective in inhibiting beating. Between days 5 and 7, sensitivity to inhibition by muscarinic agents increases to the adult level. QNB binds specifically to muscarinic acetylcholine receptors in embryonic and adult heart as assessed by competition studies with muscarinic and nicotinic agents. Receptors, as detected by QNB binding, are present in unresponsive early embryonic hearts and the number per mg heart protein does not increase dramatically during development. Receptor desensitization is accompanied by a small change (3 fold increase) in K_D for muscarinic agonists as measured by competition with ^3H QNB. This change in K_D occurs only in hearts that are responsive to muscarinic agents and thus may be associated with the physiologic action of the receptor. Competition curves for agonists suggest the involvement of negative cooperativity in activation of the receptor. These results suggest that muscarinic acetylcholine receptors, like action potential ionophores, are present in early embryonic hearts in modified "precursor" or "inactive" forms and undergo activation during development.

Significance to Biomedical Research: The results provide new insight into the developmental regulation of membrane macromolecules involved in information transfer and processing in the nervous system and in maintenance of normal beating in heart.

Proposed Course: Planned investigations include (1) studying further the effects of electrical stimulation on the properties of the action potential ionophores of skeletal and heart muscle cells in culture, (2) studying the effects of nerve on the action potential ionophore activity of skeletal and heart muscle cells in culture, (3) developing direct biochemical assay methods for action potential Ca^{++} ionophore activity and (4) studying the synthesis and degradation of the Ca^{++} ionophore and the Na^{+} ionophore using direct binding methods (see project number Z01 HL 00004-03 LBG) in heart and skeletal muscle cells at different developmental stages in culture.

Publications:

1. Catterall, W. A.: Activation and inhibition of the action potential Na^{+} ionophore of cultured rat muscle cells by neurotoxins. Biochem. Biophys. Res. Commun. 68: 136-142, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00014-01 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Storage and Release of Molecules Required for Synaptic Communication

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, Lab. of Biochem. Genetics	LBG NHLI
OTHER:	Richard McGee	Staff Fellow	LBG NHLI
	Saburo Ayukawa	Visiting Fellow	LBG NHLI
	Clifford Christian	Special Fellow	BB NICHD
	Phillip Nelson	Chief, Behavioral Biology Branch	BB NICHD

COOPERATING UNITS (if any)

Behavioral Biology Branch, NICHD

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives are to devise biochemical assays for neurotransmitter release from neuroblastoma and hybrid cell lines and then to define the steps which are required for neurotransmitter storage and release and factors which regulate these reactions.

1227

PHS-6040
(12-75)

Project Description:

Major Findings: The results show that the uptake of ^{3}H-choline into neuroblastoma x glioma hybrid cells, the rate of acetylcholine synthesis and the storage of acetylcholine are regulated by the conditions of cell growth. The results suggest that the evoked release of ^{3}H-acetylcholine can be obtained but further work is needed to clarify the release process products. The results also showed the presence of a dopamine storage mechanism in some cell lines.

Proposed Course: When the assays for transmitter release have been validated, they will be used to determine the reactions which are required for transmitter release and to determine whether the steps are regulated.

Publications:

1. Breakefield, Xandra O., Neale, Elaine A., Neale, Joseph H. and Jacobowitz, David M.: Localized catecholamine storage associated with granules in murine neuroblastoma cells. Brain Res. 92: 237-256, 1975.

2. Rotman, Avner, Daly, John W., Creveling, Cyrus R. and Breakefield, Xandra O.: Uptake and Binding of dopamine and 6-hydroxydopamine in murine neuroblastoma and fibroblast cells. Biochem. Pharmacol. 25: 383-388, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00015-01 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Regulation of cyclic nucleotide biosynthesis by neurotransmitters and opiates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, Lab. of Biochem. Genetics	LBG NHLI
	Steven L. Sabol	Research Associate	LBG NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	.5

SUMMARY OF WORK (200 words or less - underline keywords)

The role of the cyclic nucleotides adenosine 3'5' monophosphate and guanosine 3'5' monophosphate in synaptic transmission is under study using cultured cells of neural origin. The topics of interest during the current year have been the following: 1) The receptor-mediated inhibition of adenylate cyclase activity by alpha-adrenergic agents in neuroblastoma x glioma hybrid cells; 2) Demonstration of a compensatory induction of adenylate cyclase activity in cells treated for one or more days with alpha-adrenergic agents, as had been previously demonstrated by others for opiates, and study of the mechanism of this compensatory induction; 3) The effect of opiates and alpha-adrenergic agents on guanosine 3'5' monophosphate levels in the hybrid cells; 4) Search for a cultured-cell system which synthesizes a peptide with opiate-like properties.

1229

PHS-6040
(12-75)

Project Description:

Objectives: Previous work in this laboratory demonstrated that opiates, α-adrenergic agonists, and muscarinic cholinergic agonists lower cyclic AMP (adenosine 3'5' monophosphate) levels by receptor-mediated mechanisms in the neuroblastoma x glioma hybrid cell line NG108-15. Also demonstrated was the inhibition of adenylate cyclase by opiates and a compensatory induction of adenylate cyclase activity (measured in the absence of opiates) in cells grown in the presence of opiates. One aspect of the present project attempts to demonstrate and analyze similar effects of adrenergic agonists on adenylate cyclase in cell-free systems. Another aspect is to relate these findings to the action of the enkephalins, recently discovered endogenous opiate-like peptides.

Major Findings: (1) Adrenergic agents, like opiates, inhibit adenylate cyclase activity in NG108-15 homogenates. The order of agonist potencies is consistent with an alpha receptor, and the effect is blocked by alpha receptor antagonists.

(2) Cultivation of NG108-15 with norepinephrine for 1 to 4 days results, as in the case with opiates, in an increase in adenylate cyclase activity which compensates for the inhibition of this enzyme by norepinephrine. Cells then appear tolerant to norepinephrine, but are dependent upon norepinephrine for maintenance of normal cAMP levels. Withdrawal of norepinephrine or the addition of an alpha-adrenergic antagonist results in an increase in cellular cyclic AMP levels to abnormally high values.

Significance to Biomedical Research: The results show that cells exposed to α-receptor activators for several days become tolerant to and dependent upon these compounds and that tolerance and dependence are established by the mechanism of dual regulation of adenylate cyclase.

Proposed Course: It is intended to continue the investigation of the induction of adenylate cyclase activity by cyclase inhibitors, including the possible mediation of cyclase activators. Also the relationship among the opiate, adrenergic and cholinergic receptors in NG108-15 should be studied. Finally a search for endogenous "endorphin" synthesis in cell lines of neural origin is being initiated, with the goal of developing a tissue culture system for the study of the biosynthesis of this peptide or peptides.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00016-01 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptor-Mediated Regulation of Adenylate Cyclase in Hybrid Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, Lab. of Biochem. Genetics	LBG NHLI
	Neil M. Nathanson	Guest Worker	LBG NHLI

COOPERATING UNITS (if any)

Muscular Dystrophy Association

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.05	1.05	

SUMMARY OF WORK (200 words or less - underline keywords)

The activity of adenylate cyclase in neuroblastoma x glioma hybrid NG108-15 cells is regulated by the interaction of acetylcholine and its analogues with the muscarinic acetylcholine receptors of the cells. Addition of the acetylcholine analogue carbamylcholine inhibits adenylate cyclase activity. However, growth of cells with carbachol results in a prolonged increase in adenylate cyclase activity. Adenylate cyclase activity increases slowly and after 18-24 hours is 1.5-3 times higher than control values. Intracellular cAMP is also 1.5-3-fold higher in carbachol-grown cells than in controls. These changes are maintained for at least three days in the presence of carbachol, but the activity returns to control values if carbachol is removed.

1231

PHS-6040
(12-75)

Project Description:

Objectives: The objective of this project is to study muscarinic acetylcholine-receptor mediated regulation of adenylate cyclase activity, in an attempt to understand how neurotransmitter-receptor interactions modulate synaptic transmission.

Major Findings: The activity of adenylate cyclase in neuroblastoma x glioma hybrid NG108-15 cells is regulated by the interaction of acetylcholine and its analogues with the muscarinic acetylcholine receptors of the cells. Addition of the acetylcholine analogue carbamylcholine inhibits adenylate cyclase activity. However, growth of cells with carbachol results in a prolonged increase in adenylate cyclase activity. Adenylate cyclase activity increases slowly and after 18-24 hours is 1.5-3 times higher than control values. Intracellular cAMP is also 1.5-3-fold higher in carbachol-grown cells than in controls. These changes are maintained for at least three days in the presence of carbachol, but the activity returns to control values if carbachol is removed.

Significance to Biomedical Research: The results obtained suggest that neurotransmitter-receptor interactions can exert long-lived effects on macromolecules required for synaptic transmission.

Proposed Course: Work on this project will be incorporated into other projects in the coming year.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00017-01 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptors in the Developing Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, LBG	LBG NHLI
	Hiroyuki Sugiyama	Research Associate	LBG NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	.3

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to define the properties of muscarinic and nicotinic acetylcholine receptors of chick embryo retina before and after synaptogenesis in the retina. Thus far we have elucidated (1) the specificity and affinities of muscarinic and nicotinic acetylcholine receptors for cholinergic agonists and antagonists, (2) the number of receptors were defined as a function of developmental age of the retina, and (3) the location of nicotinic receptors within the retina was determined.

1233

PHS-6040
(12-75)

Project Description:

Objectives: The objectives are to define the biochemical properties of acetylcholine receptors before and after synaptogenesis in the retina.

Major Findings: Neurons dissociated from chick embryo retina and maintained in vitro were found to reaggregate and form, in vitro, approximately 1×10^9 synapses per mg of protein. Three types of synapses and several subtypes were identified which closely resemble those of the intact retina.

Chick embryo retina was found to be a rich source of both muscarinic and nicotinic acetylcholine receptors. Both muscarinic and nicotinic acetylcholine receptors are synthesized before synapses appear in the retina; however, during development, nicotinic acetylcholine receptors become associated predominantly with neurites in the synaptic layers of the retina. The properties of muscarinic acetylcholine receptors were determined at different developmental ages and were compared with the properties of muscarinic inhibitory and excitatory receptors of neuroblastoma cells.

Significance to Biomedical Research: This study gives fundamental information and serves as a basis for further studies on the role of this receptor protein in synapse formation.

Proposed Course: More detailed studies of synaptogenesis and receptor properties are planned.

Publications:

1. Vogel, Zvi and Nirenberg, Marshall: Localization of acetylcholine receptors during synaptogenesis in retina. Proc. Natl. Acad. Sci. USA, In Press.

2. Vogel, Zvi, Daniels, Mathew P. and Nirenberg, Marshall: Synapse and acetylcholine receptor synthesis by neurons dissociated from retina. Proc. Natl. Acad. Sci., USA, In Press.

3. Gouras, P., Chader, G., Enrigues, N. and Gibbons, R.G.: Calcium-induced spikes in cultures pigment epithelium of chich retina. Invest. Ophthalmol. 15: 62-64, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00018-01 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Cell Surface Features of Regenerating Retinal Neurons in Goldfish

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Greg J. Maloney, Guest Worker, LBG NHLI
OTHER: Mathew P. Daniels, Staff Fellow, LBG NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

SUMMARY OF WORK (200 words or less - underline keywords)

This work seeks to understand the role that the cell surface plays in the regeneration and reestablishment of synaptic connections in the goldfish visual system. Goldfish retinal cells have been dissociated by trypsin and have been grown in tissue culture. The neurites will be examined with electron microscopy for synapse formation. The dissociated and cultured cells will be examined for agglutinability with lectins at different stages of the regeneration response.

1235

PHS-6040
(12-75)

Project Description:

Objectives: The objectives of this work are to identify changes in the cell surfaces of retinal and tectal neurons which may be linked to the regenerative capacities of these cells. This information may lead to a better understanding of nerve growth and differentiation and to more successful techniques for treating nerve injury. The methods used are those of standard electron microscopy, tissue culture, and cell agglutination.

Major Findings: A major finding to date has been the ability to obtain viable dissociated adult retinal neurons from goldfish and to grow them in tissue culture.

Significance to Biomedical Research: The significance of this research is that it will analyze CNS nerve regeneration in terms of interactions between the surfaces of the cells and is thus related to the general problem of cell differentiation and recognition in addition to the specific area of neuron recovery after injury.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00151-06 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

The Biology of Cyclic Nucleotides in E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Peterkofsky	Deputy Chief, Lab. of Biochem. Genetics	LBG NHLI
	James P. Harwood	Staff Fellow	LBG NHLI
	Jose E. Gonzalez	Staff Fellow	LBG NHLI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	1

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long-range purpose of this project to understand the biological role and mechanisms regulating the nucleotides, cyclic AMP and cyclic GMP in Escherichia coli. The interests during the current year were: 1) to develop a system whereby the adenylate cyclase of E. coli could be studied in a manner that preserved its repression properties; 2) to study the interrelatedness of E. coli adenylate cyclase with carbohydrate transport systems; 3) to outline the profile of cyclic GMP accumulation during the life cycle of E. coli.

1237

Project Description:

Major Findings: Our previous studies concerned with elucidating the biochemical basis for catabolite repression in E. coli have led us to concentrate our attention on the sugar-dependent inhibition of adenylate cyclase. We have now found that toluene treatment of Escherichia coli B makes it possible to measure adenylate cyclase directly using [α-^{32}P]-ATP as substrate. In contrast to the non-repressible properties of adenylate cyclase in broken cell preparations, the toluene treated cells are inhibited by glucose. Cells permeabilized with toluene exhibit glucose inhibition at 10 μM, in a reaction that is rapid in onset and readily reversible. Since the enzyme is not inhibited by glucose-6-phosphate, we concluded that extensive metabolism of glucose is not necessary for the inhibition. The use of cells permeabilized with toluene has allowed us to demonstrate that the expression of a high level of adenylate cyclase activity as well as repression by glucose requires the presence of potassium phosphate. The nature of the phosphate requirement has not been clearly elucidated; however, since potassium phosphate does not stimulate adenylate cyclase activity in broken cell preparations, we believe that it may play a role in the function of some multienzyme complex that is maintained in intact and toluene-treated cells, but is disrupted in broken cells.

A number of sugars besides glucose can lead to adenylate cyclase inhibition, provided the transport systems for those sugars are induced. This correlation of the presence of a transport system for a particular sugar with the capacity of that sugar to inhibit adenylate cyclase has prompted us to examine the properties of some mutants in the sugar transport system called the phosphoenolpyruvate:sugar phosphotransferase (PTS) system, which is described by the following reactions:

1) phosphoenolpyruvate (PEP) + HPr $\xrightarrow{\text{enzyme I}}$ P-HPr

2) P-HPr + sugar $\xrightarrow[\text{(Sugar Specific)}]{\text{Enzyme II}}$ sugar-phosphate

The proteins Enzyme I and HPr are soluble and show no sugar specificity, while there is a family of enzymes II that is membrane-bound and sugar-specific. Measurements of cAMP levels in actively growing cells indicated that an HPr mutant showed normal cAMP levels, but that a leaky Enzyme I mutant showed markedly depressed levels of cAMP. The unique property of the Enzyme I mutant was also observed in measurements of adenylate cyclase activity in permeabilized cells: the Enzyme I mutant had low adenylate cyclase activity while the HPr mutant had normal enzyme levels. Since measurement of the enzyme activity in intact cells under carbon source starvation conditions indicated that the Enzyme I mutant had high adenylate cyclase activity, we concluded that the property of this mutant was an unusual sensitivity of the adenylate cyclase to variations in carbon source. We concluded that some phosphorylation mechanism may be involved in the regulation of adenylate cyclase when we observed that the addition of phosphoenolpyruvate to permeabilized cell preparations of the Enzyme I mutant led to a substantial activation of the enzyme. On the basis of these results, we have formulated the working hypothesis that Enzyme I of the PTS system plays an important role in regulating adenylate cyclase

activity. We are attempting to test the model that Enzyme I interacts with adenylate cyclase and that the PEP-dependent phosphorylation of Enzyme I is responsible for enzyme activation.

As a preliminary approach to mapping out the molecular biology of cyclic GMP in E. coli, we have carried out a variety of experiments to test the hypothesis, recently proposed by Goldberg, that cGMP levels vary in a sense opposite to those of cAMP. Our studies indicate that this hypothesis does not hold under all conditions, but rather that cGMP levels vary in ways apparently unrelated to that of cAMP. The results showed: 1) Cells starved for carbon source for a short time period had high cAMP and low cGMP concentrations. Addition of carbon source led to a decrease in cAMP and an increase in cGMP (a bi-directional change). 2) Washed cells starved for carbon source for long time periods had low cAMP levels which did not respond to the addition of glucose; however, the addition of glucose led to a transient increase in the level of cGMP (an uncoupled change). 3) Sham shift-up experiments (addition of α-methylglucoside to cells growing in succinate medium) with two strains of E. coli showed decreases in cGMP in both strains; on the other hand, cAMP increased in one strain but decreased in the other.

Significance to Biomedical Research: We have established that the enzymatic basis for the phenomenon of catabolite repression involves a sugar-dependent inhibition of adenylate cyclase. Our demonstration that we can maintain the enzyme in a repressible form has allowed a further understanding of the system. Experiments with the permeabilized cell system have permitted us to demonstrate that potassium phosphate, phosphoenolpyruvate and at least one of the enzymes involved in sugar transport are hitherto undetected important elements in the regulation of adenylate cyclase. Additional studies along these lines may further our understanding of the mechanism of receptor interactions with membrane-bound adenylate cyclase.

Proposed Course: We plan to continue our analysis of the mechanism by which sugars regulate adenylate cyclase, taking advantage of the tools of microbial genetics. Our studies involved with the biochemistry of cyclic GMP will also continue.

Publications:

1. Harwood, J. P. and Peterkofsky, A.: Glucose-sensitive adenylate cyclase in toluene-treated cells of Escherichia coli B. J. Biol. Chem. 250: 4656-4662, 1975.

2. Peterkofsky, A. and Gazdar, C.: Interaction of enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system with adenylate cyclase of Escherichia coli. Proc. Natl. Acad. Sci. USA 72: 2920-2924, 1975.

3. Gonzalez, J. E. and Peterkofsky, A.: Diverse directional changes of cGMP relative to cAMP in E. coli. Biochem. Biophys. Res. Commun. 67: 190-197, 1975.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00152-03 LBG

PERIOD COVERED
July 1, 1975 through June 30, 1976

TITLE OF PROJECT (80 characters or less)

Mechanism in Protein Synthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Peterkofsky	Deputy Chief, Lab. of Biochem. Genetics	LBG NHLI
	Chandan Prasad	Staff Fellow	LBG NHLI
	M. Fournier	Assistant Professor, Department of Biochemistry	University of Massachusetts
	J. Craine	Staff Fellow	LB NCI

COOPERATING UNITS (if any)
Department of Biochemistry, University of Massachusetts
Laboratory of Biochemistry, NCI

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

INSTITUTE AND LOCATION
NHLI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

SUMMARY OF WORK (200 words or less - underline keywords)

It is the goal of this project to increase our understanding of several unsolve problems in the field of the synthesis and regulation of proteins. Our focus of interest during the past year was two-fold. Firstly, we continued our examination of the nature of the regulation of the synthesis of transfer ribonucleic acid in Escherichia coli. Secondly, we further pursued our efforts to understand the mechanism of the reaction catalyzed by the enzyme from Escherichia coli that attaches arginine to transfer ribonucleic acid.

1240

Project Description:

Major Findings: We undertook a study of the intracellular condition of tRNA during amino acid deprivation. Since specific isoacceptor tRNA's are involved in amino-acid mediated repression and end-product inhibition, we set out to explore whether the tRNA in amino acid-starved wild-type cells and certain regulatory mutants would show compositional differences. We found that, in relaxed control E. coli, tRNA isoacceptor changes do indeed occur under conditions of amino acid starvation. Leucine starvation results in the formation of new isoacceptor species of leucine, histidine, arginine, valine and phenylalanine-specific tRNA and quantitative changes in the concentration of some other isoacceptors. Experiments with stringent strains or the use of uracil stravation or rifampin addition provided evidence for the de novo synthesis of new species of isoacceptor tRNA. Heating experiments showed that new species of leucine tRNA are not aggregation artifacts or nuclease-damaged forms of a normal leucine isoacceptor. Tests of methyl acceptor capacity of the new form of leucine tRNA indicated that a gross deficiency of methylation could not explain the properties of the unique form of leucine tRNA. Since there is some evidence from the recent work of others that tRNA formed under conditions of amino-acid starvation is deficient in the minor bases 5,6-dihydrouridine and 4-thiouridine, we think that the biochemical explanation for the accumulation of new tRNA species under conditions of amino-acid starvation may be that the enzymes responsible for these tRNA modifications are unstable and require continued protein synthesis to maintain their levels of activity.

We have continued our study of the reaction mechanism of the enzyme arginyl-tRNA synthetase, which has a mechanism atypical of most aminoacyl-tRNA synthetases. It does not catalyze an amino-acid dependent ATP-PPi exchange in the absence of added tRNA as do most enzymes of this class. In order to clarify the reaction mechanism by performing experiments with substrate levels of enzyme, we have developed a new purification procedure in which we can make homogeneous enzyme in approximately 10% yield. Pulse-labeling experiments indicate that no enzyme-bound arginyl-adenylate is formed in the absence of added tRNA. Equilibrium experiments show that no arginyl-adenylate accumulates either in the presence or absence of $tRNA^{arg}$. These data further validate our earlier suggestion that the mechanism of this reaction is probably concerted.

A series of additional studies carried out with the pure preparation of arginyl-tRNA synthetase from E. coli indicate that metals may have two functional roles in the catalytic mechanism. Complete metal activation is observed when $MgCl_2$, $MnCl_2$, $CoCl_2$, or $FeCl_2$ is present at a concentration (5 mM) in excess of the total ATP concentration (2 mM). When $CaCl_2$ is substituted for $MgCl_2$, activity is not observed unless a small amount (0.1 mM) of $MgCl_2$, $MnCl_2$, $CoCl_2$, $FeCl_2$ or $ZnCl_2$ is added. On the basis of these experiments, we visualize a model in which the enzyme possesses a site for free metal which, when filled, lowers the Km for all three substrates (arginine, $tRNA^{arg}$, and metal·ATP) and increases the V_{max} of the reaction.

Significance to Biochemical Research: 1) The study on the formation of new classes of tRNA under conditions where protein synthesis is blocked pro-

vides further insight into the interlocking regulation between RNA and protein synthesis. The lability of the proteins involved in post-transcriptional modification of tRNA may be an important locus of this regulation. 2) We have further elucidated the reaction mechanism of an unusual member of an important class of enzymes, the aminoacyl-tRNA synthetases. Our studies show that those enzymes that do not catalyze an ATP-PPi exchange in the absence of added tRNA probably proceed by a concerted mechanism. We further present new information that metals can play an allosteric regulatory role in the mechanism of these enzymes.

Proposed Course: We will continue our analysis of regulatory mechanisms in protein and RNA synthesis in E. coli.

Publications:

1. Fournier, M. J. and Peterkofsky, A.: Formation of chromatographically unique species of transfer ribonucleic acid during amino acid starvation of relaxed control Escherichia coli. J. Bacteriol. 122: 538-548, 1975.

2. Craine, J. and Peterkofsky, A.: Evidence that arginyl-adenylate is not an intermediate in the arginyl-tRNA synthetase reaction. Arch. Biochem. Biophys. 168: 343-350, 1975.

3. Craine, J. E. and Peterkofsky, A.: Studies on arginyl-tRNA synthetase from Escherichia coli B. Dual role of metals in enzyme catalysis. J. Biol. Chem. 251: 241-246, 1976.

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